

**CHARACTERIZATION OF FACTORS THAT IMPACT APOLIPOPROTEIN B
SECRETION AND ENDOPLASMIC RETICULUM ASSOCIATED DEGRADATION**

by

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CHARACTERIZATION OF FACTORS THAT CONTRIBUTE TO APOLIPOPROTEIN B SECRETION AND DEGRADATION

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University of Pittsburgh, 2013

Apolipoprotein B (ApoB) is a lipoprotein that transports cholesterol and triglycerides through the bloodstream. High plasma levels of ApoB are one of the strongest risk factors for the development of Coronary Artery Disease. Using a yeast expression system for ApoB, I focused my research on identifying new therapeutic targets to reduce the amount of ApoB secreted into the bloodstream. One way that ApoB levels are regulated is through Endoplasmic Reticulum-Associated Degradation (ERAD), a quality control mechanism that rids the secretory pathway of misfolded proteins. Due to ApoB's hydrophobic character and high number of disulfide bonds, one class of proteins that I hypothesized may contribute to ApoB ERAD was the Protein Disulfide Isomerase (PDI) family. PDI's catalyze the oxidation, reduction, and isomerization of disulfide bonds and some also have chaperone-like activity. I found that in yeast, Pdi1 contributes to ApoB ERAD through its chaperone like domain. I identified mammalian PDI candidates that may similarly affect ApoB biogenesis based on my yeast data. I found that in hepatic cells, two PDI family members, ERp57 and ERp72, contribute to ApoB ERAD, while another family member, PDI, promoted ApoB secretion.

A unique aspect of ApoB ERAD is that the protein is co-translationally retrotranslocated and degraded. I hypothesized that proteins that regulate the Sec61 translocon, a proteinaceous channel that allows ApoB entrance to the ER as well as an exit to the cytoplasm for degradation, would contribute to ApoB retrotranslocation and degradation. I discovered that two conserved

ER-membrane proteins that are candidates for Sec61 regulators, Yet2 and Yet3, facilitate the ERAD of ApoB in yeast. To determine whether my results are relevant in mammalian cells, I am currently working to determine if the mammalian homologs of Yet2 and Yet3, BAP29 and BAP31 facilitate ApoB ERAD in hepatic cells.

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PREFACE

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LIST OF FREQUENTLY USED ABBREVIATIONS

ApoB – Apolipoprotein B

ERAD – Endoplasmic Reticulum – Associated Degradation

PDI – Protein Disulfide Isomerase

VLDL – Very Low Density Lipoprotein

LDL – Low Density Lipoprotein

HDL – High Density Lipoprotein

TAG - Triacylglycerol

DAG - Diacylglycerol

FFA – Free Fatty Acids

LPL – Lipoprotein Lipase

LDLR – LDL Receptor

MTP – Microsomal Triglyceride Transfer Protein

LD – Lipid Droplet

SRP – Signal Recognition Particle

SR – SRP Receptor

SS – Signal Sequence

PERPP – Post-ER Presecretory Proteolysis

1.0 INTRODUCTION

1.1 LIPID CARRIERS – CHYLOMICRONS, VLDL, LDL, HDL

Mammals have three sources of fatty acids and cholesterol – those that are synthesized, obtained through the diet, and stored in cells. The synthesis of cholesterol occurs primarily in the liver, where a small fraction is incorporated into hepatocyte membranes (Goldstein and Brown, 1977). The cholesterol that is not incorporated into membranes in the liver is exported as biliary cholesterol, bile acids, or cholesteryl esters to be incorporated into membranes in other tissues or used as a precursor for steroid hormones and vitamin D (Soccio and Breslow, 2004). Cholesterol and cholesteryl esters are hydrophobic and therefore insoluble. In order to maintain cellular homeostasis, lipoproteins, composed of different apolipoproteins and combinations of phospholipids, triacylglycerols (TAG), cholesterol, and cholesteryl esters, are necessary to transport these hydrophobic molecules through the bloodstream from the liver, where they are synthesized, or from the small intestines, where they are absorbed from dietary nutrients, to tissues where they will be used or stored (Goldstein and Brown, 1977; Kwiterovich, 2000).

There are several different classes of lipoproteins - chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) (Figure 1) (Kwiterovich, 2000). These lipoproteins have specific functions based on where they are synthesized, their lipid composition, and the apolipoprotein content, and they can be

separated from the plasma by ultracentrifugation based on their densities (Dominiczak and Caslake, 2011). Apolipoproteins are amphipathic lipid-binding proteins that act as detergents to transport TAG, phospholipids, cholesterol, and cholesteryl esters through the bloodstream. At least nine different apolipoproteins can be found in lipoproteins isolated from human plasma. In addition to acting as detergents, apolipoproteins can function as signal transducers by targeting specific lipoproteins to receptors on various tissues or they can activate enzymes that act on lipoproteins (Dominiczak and Caslake, 2011).

The second source of fatty acids and cholesterol is dietary. For example, humans obtain a significant portion of their daily energy requirement through dietary TAG, which are absorbed in the small intestine. In the small intestine, TAG are converted from insoluble fat molecules to mixed micelles by bile salts, which are synthesized in the liver from cholesterol, stored in the gall bladder, and released into the small intestine after ingestion of a meal rich in fat (Abumrad and Davidson, 2012). The mixed micelles are broken down in the small intestines by lipases converting TAG into monoglycerides, diacylglycerols (DAG), free fatty acids (FFA), and glycerol (Abumrad and Davidson, 2012). These breakdown products are absorbed into the intestinal epithelial cells by diffusion where they are re-converted into TAG, packaged with dietary cholesterol and specific apolipoproteins, such as ApoB48 and ApoC-II, to form chylomicrons, a class of lipoproteins characterized by an extremely low density and large diameter (Mansbach and Siddiqi, 2010).

For dietary fats to be utilized by the body, chylomicrons traffic from the intestinal mucosa into the lymphatic system, and then into the blood stream where they are carried to muscle and adipose tissue (Mansbach and Siddiqi, 2010). Once in these tissues, apolipoprotein ApoC-II activates the enzyme Lipoprotein Lipase (LPL), which hydrolyzes TAG to FFA and

glycerol that can be utilized by the cells (Havel, 1975; Mjos *et al.*, 1975; Kwiterovich, 2000). After being depleted of TAG the chylomicron remnants contain cholesterol, and the apolipoproteins ApoB48 and ApoE, and travel to the liver where they are endocytosed, and degraded by the lysosome (Kita *et al.*, 1982; Rubinsztein *et al.*, 1990; Rohlmann *et al.*, 1998).

FFA in liver cells are converted to TAG, which gets packaged with phospholipids, cholesterol, cholesteryl esters, and specific apolipoproteins, to form nascent VLDL particles (Goldstein and Brown, 1977; Dominiczak and Caslake, 2011). The key apolipoprotein in VLDLs is ApolipoproteinB100 (ApoB100). VLDLs are secreted into the bloodstream and travel to muscle and adipose tissue where ApoC-II activates LPL, causing the release of FFA from the TAG (Havel, 1975; Kwiterovich, 2000; Dominiczak and Caslake, 2011). The FFA are absorbed by myocytes and adipocytes and are oxidized to provide energy or resynthesized into TAG for storage (Kwiterovich, 2000). The reduction in TAG content of the VLDL by LPL increases the density of the particle, and it is now referred to as a LDL.

LDLs are rich in cholesterol and cholesteryl esters and like VLDLs they also predominantly contain ApoB100 (Goldstein and Brown, 1977). LDLs deliver fatty nutrients to peripheral tissues that require cholesterol for new membrane synthesis. Upon conversion to an LDL, a portion of the ApoB100 polypeptide that was previously buried in a VLDL by lipids becomes exposed. The exposed portion of ApoB is the ligand for the LDL-receptor (LDLR) (Boren *et al.*, 1998), which is selectively expressed on tissues that are competent for cholesterol and FA metabolism, and is necessary for receptor mediated endocytosis of ApoB containing LDL particles (Brown and Goldstein, 1975, 1976). Upon endocytosis in target tissues, the endosome fuses with the lysosome, where ApoB is degraded and the cholesteryl esters are

hydrolyzed into cholesterol and FA. These fats are released into the cytosol and may be incorporated into membranes or re-esterified and stored (Goldstein and Brown, 1977).

The final class of lipoproteins is HDLs, which are synthesized in the liver or small intestine as protein rich particles and initially contain very little cholesterol and no cholesteryl esters (Kwiterovich, 2000). HDLs predominantly contain the apolipoproteins ApoC-I and ApoC-II and the enzyme lecithin-cholesterol acyl transferase (LCAT). LCAT is present on nascent HDLs and converts cholesterol and phosphatidylcholine from chylomicron and VLDL remnants to cholesteryl esters (Scanu and Edelstein, 2008). The cholesteryl esters form a core for the HDL particles and facilitate maturation. The mature HDLs return to the liver and deliver the acquired cholesterol for conversion into bile salts (Scanu and Edelstein, 2008).

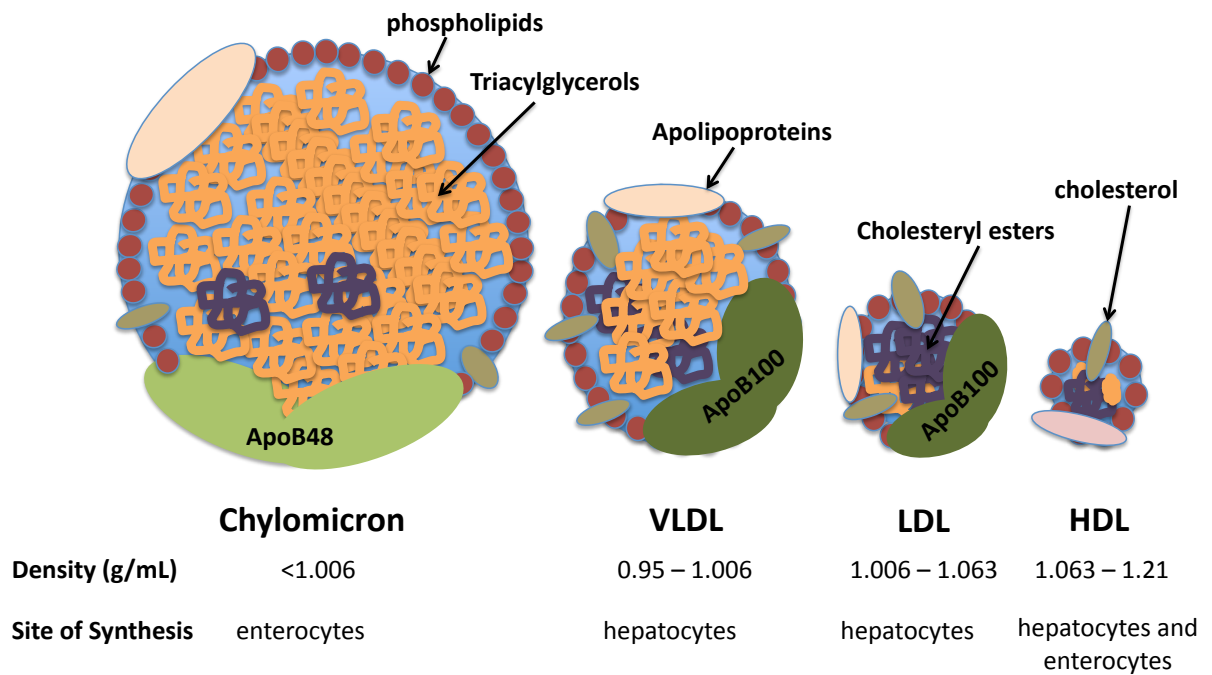


Figure 1. Major classes of human plasma lipoproteins

The four major types of plasma lipoproteins are depicted with their various components. Chylomicrons have the largest diameter and are the least dense particles. They contain the apolipoprotein ApoB48 and contain high concentrations of triacylglycerols. VLDL particles have the second largest diameter and harbor the apolipoprotein ApoB100. VLDLs contain a core of both triacylglycerols and cholesteryl esters, with a larger percentage of triacylglycerols. LDL particles are more dense than VLDLs and have a smaller diameter. They also contain the apolipoprotein ApoB100 and a core of both triacylglycerols and cholesteryl esters, with a larger percentage of cholesteryl esters. The most dense lipoprotein particles with the smallest diameter are HDLs. HDLs do not express ApoB, but are mainly composed of other apolipoproteins, such as ApoC-I and ApoC-II and phospholipids. See (Havel, 1975), for additional details.

1.2 APOLIPOPROTEIN B

Apolipoprotein B (ApoB) is a large amphipathic lipid binding protein produced in the liver and small intestine in two different isoforms – ApoB100 and ApoB48. ApoB is the main structural component of chylomicrons, VLDLs, and LDLs. The amphipathic nature of ApoB allows it to act as a detergent for the transport of the highly hydrophobic lipoproteins through the bloodstream. Collectively, chylomicrons, VLDLs, and LDLs distribute dietary and endogenously synthesized cholesterol and lipids to peripheral tissues, to be used for membrane synthesis, bile acid synthesis, and cellular metabolism. Although these lipoproteins are extremely important for maintaining cellular homeostasis by delivering cholesterol and lipids to various tissues, ApoB containing lipoproteins, with the exception of chylomicrons, are particularly atherogenic. These circulating particles can accumulate in atherosclerotic plaques in arteries, leading to coronary artery disease (Tiwari and Siddiqi, 2012). Due to the critical role of ApoB in lipoprotein transport, its biogenesis is highly regulated, both at the levels of synthesis and degradation (see section 1.4).

1.2.1 Isoforms and Structural Domains

ApoB is encoded in the human genome by a gene that is approximately 45kb (Lusis *et al.*, 1985). In hepatic cells, the gene is transcribed into a very stable 15kb mRNA that is translated into a single 4536 amino acid polypeptide with a mass of approximately 550kDa. This species is ApoB100 (Lusis *et al.*, 1985). The modeled secondary structure of ApoB100 is based on homology to the lipid associated protein lipovitellin (Segrest *et al.*, 2001), a predominant yolk

protein in vertebrate eggs. Based on sequence prediction and homology modeling, ApoB100 is proposed to have a five-domain secondary structure consisting of both alpha helical and beta sheet structures (NH₂-β_{α1}-β₁-α₂-β₂-α₃-COOH) (Figure 2) (Segrest *et al.*, 2001). The amphipathic nature of ApoB is essential for its function. The hydrophobic regions noncovalently interact with hydrophobic lipids while the hydrophilic regions are essential for maintaining solubility in the plasma. Other specific regions of the protein are important for targeting lipoprotein particles to peripheral tissues and to the liver for receptor-mediated endocytosis (see section 1.1). Sequence comparison, antibody binding experiments, and truncation analysis identified amino acid residues 3345-3381 in ApoB as the LDLR binding domain (Milne *et al.*, 1989; Law and Scott, 1990; Boren *et al.*, 1998; Segrest *et al.*, 2001). As described in section 1.1, after VLDL are converted into LDL particles, the LDLR binding domain is exposed. This helps target the lipoprotein to peripheral tissues that express the LDLR and that are competent for cholesterol metabolism.

In enterocytes, the 15kb ApoB mRNA is post-transcriptionally edited by the 27kDa enzyme ApoB mRNA editing complex-1 (apobec-1) (Chen *et al.*, 1987; Powell *et al.*, 1987). Apobec-1 binds to an AU-rich sequence five bases downstream of cytidine-6666 and deaminates cytidine-6666, converting it to a uridine (Navaratnam *et al.*, 1993). This conversion from C to U forms a UAA stop codon instead of a glutamine residue. The result is a truncated protein comprising the N-terminal 48% of full length ApoB, ApoB48 (Figure 2). As noted above, ApoB48 is the predominant component of chylomicrons (Kane *et al.*, 1980; Chen *et al.*, 1987; Lo *et al.*, 2008; Mansbach and Siddiqi, 2010). In rodents such as mice and rats, Apobec-1 is expressed in the liver in addition to the intestines, producing significant amounts of both

ApoB48 and ApoB100 in the liver (Tennyson *et al.*, 1989); however, in humans, production of the ApoB48 isoform is restricted to the small intestine.

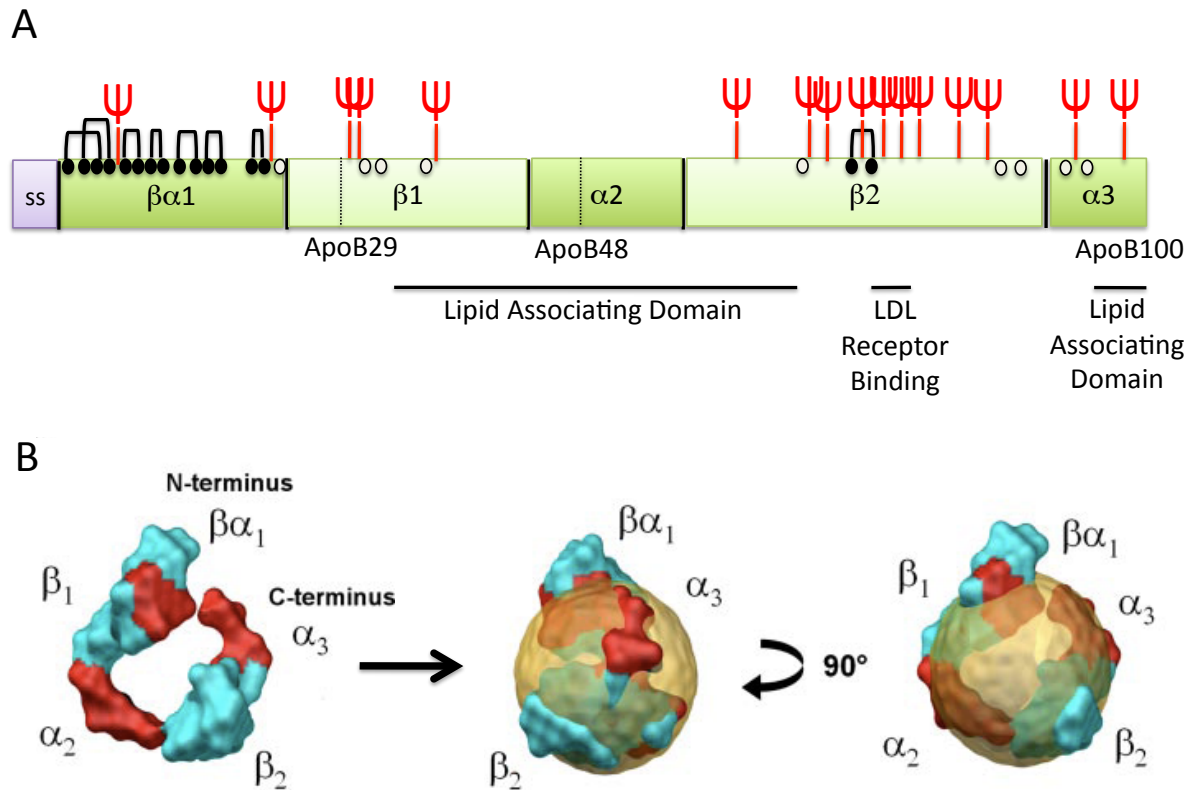


Figure 2. Isoforms and structural features of Apolipoprotein B

(A) The linear diagram of ApoB depicts the lengths of the two predominant isoforms of ApoB, ApoB48 and ApoB100, and the isoform used in the majority of my experiments, ApoB29. The C-terminus of each isoform is marked by a dashed line. The ER signal sequence (SS) consists of amino acids 1-27. ApoB100 has a total of 25 cysteines (depicted with circles) and 16 of those are involved in forming 8 disulfide bonds (dark circles) and the rest are free sulfhydryls (open circles). ApoB100 also contains 16 confirmed N-linked glycosylation sites (depicted with red pitchforks). Adapted from Harazono *et al.*, 2005. (B) The model of ApoB secondary structure in an LDL particle from Johns *et al.*, 2006 with permission. Of note, the highly disulfide bonded $\beta\alpha_1$ domain is not as tightly associated with lipids as the other domains.

Another naturally occurring isoform of ApoB that was originally identified in patients with hypobetalipoproteinemia, a disease characterized by very low LDL levels (see section 1.4.1), is ApoB29. As its name implies, ApoB29 is 29% of full length ApoB, and is 1305 amino acids in length (Figure 2). This ApoB isoform results from a C to T alteration in the ApoB gene that encodes a premature stop codon (Collins *et al.*, 1988; Huang *et al.*, 1989). Studies using ApoB29 have found that the shorter isoform limits the amount of lipids that can associate with the protein to form a lipoprotein, resulting in smaller, denser, lipid poor particles (Linton *et al.*, 1993). However, subsequent studies that investigated the ability of ApoB truncation mutants to traffic through the secretory pathway indicated that ApoB29 is the shortest isoform that is able to traffic normally (McLeod *et al.*, 1994). These results indicate that ApoB29 is the smallest clinically relevant ApoB isoform that can be studied; as such, this is the isoform that is used in the majority of my experiments.

1.2.2 Translocation and Trafficking

Synthesis of ApoB (and other secretory proteins) occurs at the ER surface after it is directed to the membrane by an N-terminal 27 amino acid signal sequence (see section 1.3.1) (Sturley *et al.*, 1994). Translocation of ApoB into the ER lumen occurs co-translationally through a proteinaceous channel in the ER membrane called the translocon. The translocon is composed mainly of the Sec61 protein, which binds tightly to ribosomes during translation and translocation into the ER (Robson and Collinson, 2006). ApoB associates with Sec61 α and Sec61 β , two components of the translocon, during its co-translational translocation (Mitchell *et al.*, 1998; Pariyarath *et al.*, 2001). As described above, ApoB contains highly hydrophobic β -

sheet domains (amino acids 1000-2000 and 2600-4000) (Figure 2), which interact strongly with the translocon as it is translated and this interaction can cause a slowing of translocation while translation continues (Ginsberg and Fisher, 2009). Translocational slowing can result in ApoB congestion in the translocon and allow loops of ApoB to be exposed to the cytosol through gaps between the ribosome and the translocon (Hegde and Lingappa, 1996). A unique feature of ApoB translation is the presence of pause transfer sequences in the polypeptide, which are clustered in three regions of the protein (9 sequence in the N-terminal 20% of ApoB100, 4 sequences between 46-50%, and 10 sequences between 65-95%)(Figure 2). These pause transfer sequences are distinct from the β -sheet domains and may also cause a slowing of ApoB translocation into the ER by causing translation to stop and restart (Chuck and Lingappa, 1992, 1993; Kivlen *et al.*, 1997); however, recent studies suggest that the hydrophobic β -sheet domains are the main contributors to this phenomenon (Yamaguchi *et al.*, 2006).

As ApoB is translocated, an ER-resident protein, the microsomal triglyceride transfer protein (MTP), composed of a 97 kDa “M” subunit and Protein Disulfide Isomerase (PDI), facilitates the transfer of phospholipids, cholesterol, and triglycerides onto ApoB, forming a primordial lipoprotein (Hussain *et al.*, 1997). PDI keeps the M subunit soluble and retained in the ER and is essential for the lipid transfer activity of MTP (Wetterau *et al.*, 1990; Wetterau *et al.*, 1991). ApoB and MTP have been shown to interact by coimmunoprecipitation (Patel and Grundy, 1996; Wu *et al.*, 1996), solid-liquid interphase binding assays (Hussain *et al.*, 1997), and yeast two hybrid assays (Bradbury *et al.*, 1999; Mann *et al.*, 1999), and this interaction occurs between the $\beta\alpha_1$ domain of ApoB and the M subunit of MTP (see Figure 2A) (Hussain *et al.*, 2003). The association of MTP with ApoB is increased upon treatment with oleic acid, as more lipid substrates are available to load onto ApoB (Homan *et al.*, 1991). Numerous studies

have indicated that the lipid transfer activity of MTP is essential for the assembly and secretion of lipoproteins containing ApoB (Gordon *et al.*, 1994; Leiper *et al.*, 1994; Jamil *et al.*, 1996; Benoist and Grand-Perret, 1997; Wang *et al.*, 1997b; Davis, 1999; Gordon and Jamil, 2000).

Lipid synthesis occurs at the ER and lipids can be sequestered and stored in cytoplasmic Lipid Droplets (LDs), which have a neutral lipid core surrounded by a phospholipid and sterol monolayer (Sturley and Hussain, 2012). LDs are often found associated with the ER forming crescents around the organelle, as it is thought that they bud from the ER membrane. They function to release lipids in response to cellular need and it is hypothesized that the lipid loading activity of MTP creates a concentration gradient that is essential for transferring neutral lipids into the ER (Sturley and Hussain, 2012). The major neutral lipid cargo in LDs is TAG and TAG stored in LDs is the predominant source of lipid substrate for the assembly of ApoB into pre-VLDLs in the ER (Yang *et al.*, 1995).

Once ApoB is successfully translocated into the ER and is assembled into a pre-VLDL upon lipid loading, it must exit the ER and traffic to the Golgi where the lipoprotein matures into a VLDL (see below). Secretory proteins normally exit the ER in COPII vesicles composed of five subunits -Sar1, Sec23/24, and Sec13/31 (Kuge *et al.*, 1994; Rothman and Wieland, 1996; Barlowe, 2000). The relatively large size of ApoB containing lipoproteins has prompted speculation that these particles may require a unique mechanism to be transported from the ER (Fromme and Schekman, 2005). Although most COPII vesicles are 60-80nm in diameter, some of these vesicles are able to increase their size to accommodate the transport of larger cargo such as procollagen, which is 300-400nm (Jin *et al.*, 2012). The mechanisms of coat size regulation have long been investigated. TANGO1 and its partner cTAGE5 were identified as proteins that interact with procollagen as well as Sec23/24 and are thought to recruit procollagen to nascent

COPII coats (Saito *et al.*, 2009; Saito *et al.*, 2011); however, it was not understood how this interaction allowed the coat to accommodate larger cargo because it was not thought that TANGO1 is a regulator of coat size. The Schekman and Rape labs recently found that the E3 ubiquitin ligase Cul3^{K1h12} monoubiquitinates the outer layer coat protein Sec31, which drives the assembly of larger COPII coats and promotes the trafficking of procollagen (Jin *et al.*, 2012). They propose that the ubiquitination event controls the size and function of the COPII coat. This mechanism may also be used to regulate that size of coats for the transport of chylomicrons and VLDLs, which the Fisher lab is currently investigating. Interestingly, studies investigating the mechanism by which ApoB containing lipoproteins are trafficked to the Golgi revealed that the ER exit process of ApoB is similar to that of other canonical cargo (Gusarova *et al.*, 2003). Specifically, ApoB trafficking was dependent on Sar1 activity, since ApoB did not exit the ER in cells expressing a dominant negative version of Sar1, and the ApoB containing vesicles contained other COPII proteins (Gusarova *et al.*, 2003). However, Siddiqi *et al.*, proposed a model that COPII proteins are not required for ER exit but are necessary for the fusion of vesicles with the Golgi (Siddiqi *et al.*, 2003). Nevertheless, mutations in Sar1 family members result in diseases such as Chylomicron Retention Disease (Roy *et al.*, 1987; Nemeth *et al.*, 1995) and Anderson Disease (Bouma *et al.*, 1986; Dannoura *et al.*, 1999), which are disorders of severe fat malabsorption and are associated with failure to thrive in infancy, suggesting that COPII vesicles are necessary to traffic chylomicrons (Jones *et al.*, 2003).

The final lipidation step, converting the ApoB containing pre-VLDL into a mature VLDL, is proposed to take place after the lipoprotein has exited the ER, either in the Golgi or in a pre-Golgi compartment (Bamberger and Lane, 1990; Stillemark *et al.*, 2000; Swift *et al.*, 2001; Tran *et al.*, 2002; Gusarova *et al.*, 2003); however, other studies provide evidence that the ER

may be the location of final VLDL assembly (Kulinski *et al.*, 2002; Yamaguchi *et al.*, 2003). Overall, ApoB shares many similarities with canonical secretory proteins with regard to its synthesis, translocation, and trafficking; however, its distinct features, such as the large size, hydrophobic domains, and pause transfer sequences, also lead to unique characteristics.

1.3 POST-TRANSLATIONAL MODIFICATIONS IN THE ENDOPLASMIC RETICULUM

Approximately one-third of all proteins traverse the secretory pathway and are either secreted or inserted into membranes to perform their specific functions (Guerriero and Brodsky, 2012). Nascent secretory proteins, such as ApoB, begin this journey in the ER where they undergo several post-translational modifications, such as signal sequence cleavage, N-linked glycosylation, and disulfide bond formation, which contribute to the protein's native fold.

1.3.1 Signal Sequence Processing

For proteins to enter the secretory pathway, they must first be directed to the ER. In order for this to occur, secretory proteins are synthesized as pre-proteins with amino-terminal extensions that act as “zipcodes” targeting the protein to the ER. Specifically, as soluble secreted proteins emerge from the exit tunnel of the ribosome, they present a cleavable signal sequence at their amino terminus that is approximately 20-30 amino acids and contains features such as a basic motif, a hydrophobic core, and a short polar region (Hegde and Bernstein, 2006). ApoB has a 27 amino acid signal sequence, which is cleaved upon entrance into the ER (Chuck and Lingappa,

1992, 1993). Mutations in the signal sequence have been linked to deficiencies in ApoB secretion causing hyperlipidemia (Sturley *et al.*, 1994).

In the cytosol, a ribonucleoprotein called the signal recognition particle (SRP) acts as a targeting factor and binds to the emerging signal sequence, which causes translational pausing (Keenan *et al.*, 2001; Wild *et al.*, 2004; Saraogi and Shan, 2011). Upon translational pausing, the entire ribosome-nascent chain complex is directed to the ER membrane where the SRP binds to a heterodimeric membrane bound SRP receptor (SR) (Powers and Walter, 1997) and the ribosome attaches to the translocation machinery in the membrane through its membrane-attachment site (M-site), forming a tight seal with the translocon (Saraogi and Shan, 2011). The SRP is then released from the signal sequence of the nascent protein and translation resumes.

As translation recommences, the nascent polypeptide is inserted into the pore of the Sec61 translocon. The nascent protein's signal sequence binds to a specific site within the Sec61 translocon, which triggers the opening of the pore. The binding of the signal sequence to this site triggering the pore opening is thought to protect aberrant proteins from entering the lumen of the ER and calcium from leaking out (Hizlan *et al.*, 2012). As the growing polypeptide chain emerges into the lumen of the ER, cleavage sites adjacent to the signal sequence, which remains bound within the pore of the Sec61 translocon, are recognized by a membrane bound signal peptidase and cleaved (Stroud and Walter, 1999). Even after cleavage, the signal sequence remains bound to the pore of the translocon while the remainder of the protein is translocated into the ER lumen. In higher eukaryotes, after the protein has been completely translocated into the ER lumen, the pore closes, and the translocon opens laterally to the lipid bilayer, allowing the signal sequence to diffuse into the bilayer where it is degraded (Weihofen *et al.*, 2002; Brodsky and Skach, 2011). A diagram of protein translocation into the ER is shown in Figure 3.

The nascent protein within the ER lumen is now exposed to a multitude of molecular chaperones. These chaperones can either aid in the post-translational modification and proper folding of the protein or recognize a protein that is terminally misfolded and target that protein for degradation. Examples of two post-translational modifications that occur within the ER are discussed in the following sections.

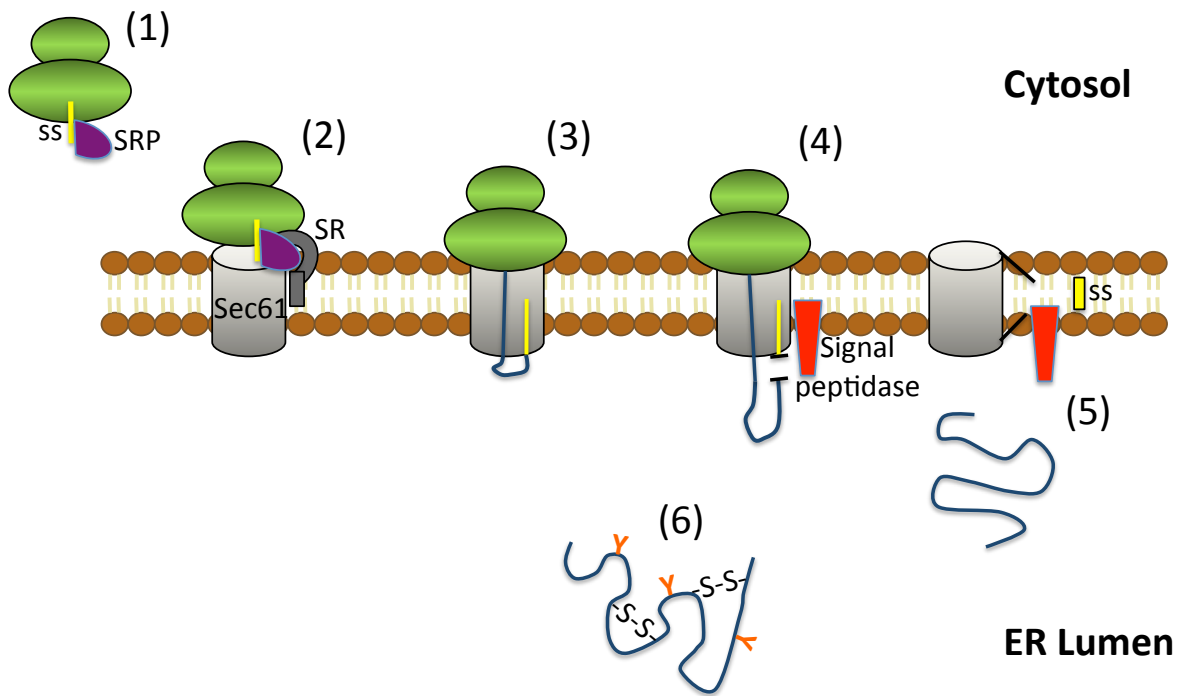


Figure 3. Soluble secretory proteins are targeted to the ER by a cleavable signal sequence

(1) A nascent polypeptide emerging from the ribosome exit tunnel exposes a signal sequence (SS in yellow) that is recognized and bound by the signal recognition particle (SRP in purple), causing translational pausing. (2) The SRP directs the entire complex to the ER membrane where it binds to a membrane bound SRP receptor (SR). (3) As translation recommences, the polypeptide translocates through Sec61 and the SS binds within the translocon. (4) The signal peptidase cleaves the SS, which remains bound in the translocon, from the rest of the polypeptide. (5) The protein fully translocates into the ER lumen and the translocon opens laterally to allow the SS to diffuse into the lipid bilayer. (6) Inside the ER lumen, the polypeptide folds into its native conformation and post-translational modifications, such as disulfide bond formation (S-S) and glycosylation (Y), take place.

1.3.2 Asparagine-linked Glycosylation

One post-translational modification that occurs within the ER lumen is the addition of sugar moieties onto asparagine residues within the consensus sequence Asn-X-Ser/Thr, termed N-linked glycosylation (Moremen *et al.*, 2012). These sugar moieties, called glycans, are transferred as preformed precursor oligosaccharides, composed of two N-acetylglucosamines, nine mannoses, and three glucoses, onto the NH₂ group of asparagines within the consensus sequence as they enter through the translocon into the lumen of the ER (Schwarz and Aeby, 2011). N-linked glycans serve numerous functions in the cell. They can act as timers for protein folding, stabilize protein structure, protect proteins from proteolysis, and mediate protein-protein interactions. Additionally, N-linked glycans facilitate the interaction of glycoproteins with the ER quality control lectins, calnexin and calreticulin (Larkin and Imperiali, 2011; Schwarz and Aeby, 2011; Moremen *et al.*, 2012).

As explained above, the sugar moiety added to asparagine residues on nascent proteins is a precursor oligosaccharide. Once the 14-sugar chain has been added to the protein, an enzymatic trimming reaction catalyzed by glucosidase I and II cleaves two of the three glucose residues (Deprez *et al.*, 2005). Following this cleavage reaction, the ER quality control lectins calnexin and calreticulin can bind to glycans on proteins that are incompletely folded, retaining the proteins in the ER and allowing them time to achieve their correct folded conformation (Aeby *et al.*, 2010). The interaction of the lectins with the glycoprotein is maintained until the third glucose is trimmed from the chain by ER glucosidase II and the protein is released (Sousa and Parodi, 1995). At this point, an evaluation of the folded state of the protein is made by the

enzyme UDP-glucose:glycoprotein glycosyltransferase. If the protein has not achieved its folded state, the enzyme will add a glucose back to the chain, renewing the affinity for calnexin and allowing it more time to remain in the ER and fold properly. This cycle can continue until the protein has reached its folded conformation (Larkin and Imperiali, 2011; Schwarz and Aeby, 2011; Moremen *et al.*, 2012). However, if the protein does not successfully fold within a timeframe, ER mannosidases remove mannose residues from the chain preventing re-entry into the calnexin cycle and targeting the protein for degradation by ER-Associated Degradation (ERAD) (Quan *et al.*, 2008), which is described in section 1.4.2.2. Among the ER mannosidases is the ER enhancing α -mannosidase-like protein I (EDEMI), which has been proposed to interact with specific glycan conformations to trim the mannoses, designating the protein for degradation (Oda *et al.*, 2003; Cormier *et al.*, 2009). Interestingly, EDEMI has also been shown to interact with misfolded substrates regardless of their glycosylation state.

Sixteen known genetic diseases, referred to as congenital disorders of glycosylation, arise from incorrect assembly or processing of N-linked glycans, which highlights the importance of N-linked glycosylation in human health (Jaeken, 2010; Guerriero and Brodsky, 2012). Additionally, the necessity of N-linked glycosylation in cellular homeostasis is evidenced by pharmacological manipulation of the pathway using the metabolite tunicamycin to inhibit early steps in the assembly of glycan chains. When glycosylation is inhibited with tunicamycin, misfolded proteins accumulate in the ER causing an induction of the Unfolded Protein Response (UPR) (Prescher and Bertozzi, 2006), an adaptive signaling cascade to re-establish ER homeostasis. Induction of the UPR has been implicated in a number of diseases from diabetes and neurodegeneration to a variety of cancers.

ApoB is subject to posttranslational modifications in the ER as it is folded into its native conformation and accumulates lipids to form pre-VLDLs. N-linked glycosylation is one modification that affects the folding and secretion of ApoB. ApoB100 has 19 putative N-glycosylation sites, and 16 of those have been confirmed to be glycosylated *in vivo* (Figure 2) (Yang *et al.*, 1989). The C-terminal β 2 domain of ApoB100 is highly glycosylated, containing 10 of the 16 utilized glycosylation sites (Harazono *et al.*, 2005). Several laboratories have investigated the role of these carbohydrate moieties on ApoB physiology. Proteasomal and non-proteasomal degradation of ApoB were both enhanced when a human hepatocyte cell line was treated with the N-glycosylation inhibitor tunicamycin (Liao and Chan, 2001). Studies on truncation mutants of ApoB containing asparagine to glutamine mutations showed decreased secretion efficiency and reduced lipid-binding affinity of the mutants (Vukmirica *et al.*, 2002). These studies indicate that efficient N-linked glycosylation is essential for secretion of the lipoprotein particle.

1.3.3 Disulfide Bond Formation

A second post-translational modification that occurs within the oxidizing environment of the ER lumen is disulfide bond formation. Disulfide bonds form through the oxidation of free thiol groups of two cysteine residues, either within one protein (intramolecular disulfide bond) or between cysteines from two different proteins (intermolecular disulfide bonds) (Hatahet and Ruddock, 2009). Intramolecular disulfide bonds provide proteins structural stability and help proteins achieve the correct folded conformation. Intermolecular disulfide bonds function to maintain the structure of oligomeric protein complexes (Hatahet and Ruddock, 2009). Improper

formation and maintenance of disulfide bonds can lead to diseases, such as Marfan syndrome (Whiteman and Handford, 2003) and von Willebrand disease (Schneppenheim *et al.*, 1996).

As described in section 1.2.1, ApoB100 is a cysteine rich, secreted protein that is subject to disulfide bond formation upon translocation into the ER lumen. ApoB100 has 25 cysteines, 16 of which are linked by intramolecular disulfide bonds (Figure 2) (Yang *et al.*, 1989; Yang *et al.*, 1990). Interestingly, the N-terminal $\beta\alpha 1$ domain of ApoB (approximately 21% of full length ApoB100) contains 14 of the 25 cysteines, all of which are disulfide bonded, accounting for 7 of the 8 disulfide bonds found in full length ApoB100 (Yang *et al.*, 1990; Burch and Herscovitz, 2000). The high disulfide bond content is predicted to make this region highly compact, which correlates with structural data (Segrest *et al.*, 2001; Johs *et al.*, 2006). Formation of disulfide bonds is critical for maturation of ApoB (Huang and Shelness, 1997; DeLozier *et al.*, 2001) and when disulfide bond formation is inhibited using the reagent dithiothreitol (DTT), ApoB is not secreted (Burch and Herscovitz, 2000). In addition, mutations in cysteines 7 and 8 that form a disulfide bond in ApoB have been shown to diminish VLDL assembly and secretion (Tran *et al.*, 1998; DeLozier *et al.*, 2001), highlighting the necessity of this posttranslational modification for homeostasis.

1.3.3.1 Protein Disulfide Isomerases

A family of enzymes known as Protein Disulfide Isomerases (PDIs), named for the founding member of the family, PDI, catalyze the formation of disulfide bonds in the ER (Hatahet and Ruddock, 2009). In addition to catalyzing oxidation reactions, PDIs also catalyze reduction and isomerization reactions, and some family members have chaperone activity (Cai *et al.*, 1994; Song and Wang, 1995; Klappa *et al.*, 1998; Gillece *et al.*, 1999; Ellgaard and Ruddock, 2005; Tian *et al.*, 2006; Tian *et al.*, 2008; Hatahet and Ruddock, 2009). Thus, PDIs are able to

make, break, and rearrange disulfide bonds to help proteins achieve their correct folded conformation, since in their native state proteins tend to only have one pattern of disulfide bonds and free thiol groups. PDIs catalyze these reactions through thiol groups on cysteine residues within their thioredoxin-like active sites, which have the consensus sequence Cys-X-X-Cys (Edman *et al.*, 1985). The two cysteines in the active site are able to cycle between an oxidized (S-S) and reduced (-SH HS-) state to interact with disulfides and free thiols on substrate proteins (Figure 4) (Hatahet and Ruddock, 2009). In yeast, PDI is able to re-enter into cycles of oxidation and reduction with substrates by being reduced or oxidized by glutathione (Chakravarthi and Bulleid, 2004; Molteni *et al.*, 2004) and the protein Ero1 (Mezghrani *et al.*, 2001), both of which are present in the ER. In mammals, two Ero1 enzymes exist, Ero1 α and Ero1 β , which serve as the primary oxidases for PDI (Araki and Inaba, 2012). Although yeast Ero1 is an essential protein, Ero1 α and Ero1 β are dispensable in mammals. Surprisingly, homozygous mice with almost complete loss-of-function mutations in Ero1 α and Ero1 β (Ero1 $\alpha^{i/i};\beta^{i/i}$) are viable (Zito *et al.*, 2010a), which suggests that in mammals Ero1 independent oxidative pathways exist (Araki and Inaba, 2012). Candidates for members of an alternative oxidative pathway include Prx4 (Tavender and Bulleid, 2010), which metabolizes H₂O₂ by reducing it to water, and the sulfhydryl oxidoreductases QSOX (Zito *et al.*, 2010b).

PDI, the founding member of the PDI family (Goldberger *et al.*, 1964), has two thioredoxin-like active site domains, denoted the a and a' active site, each with the amino acid sequence CGHC (Edman *et al.*, 1985; Darby *et al.*, 1996; Freedman *et al.*, 1998; Alanen *et al.*, 2003). The a and a' active sites are separated by two other domains that have similarity to thioredoxin, a small cytoplasmic enzyme, which is involved in thiol-dependent redox reactions (Funato and Miki, 2007; Lemaire *et al.*, 2007; Lillig and Holmgren, 2007; Hatahet and Ruddock,

2009). However, these other domains, denoted the b and b' domains, lack enzymatic activity. The b' domain provides a hydrophobic binding pocket for substrates that is responsible for PDI's chaperone activity (Cai *et al.*, 1994; Song and Wang, 1995; Klappa *et al.*, 1998; Gillece *et al.*, 1999; Ellgaard and Ruddock, 2005; Tian *et al.*, 2006; Tian *et al.*, 2008; Hatahet and Ruddock, 2009).

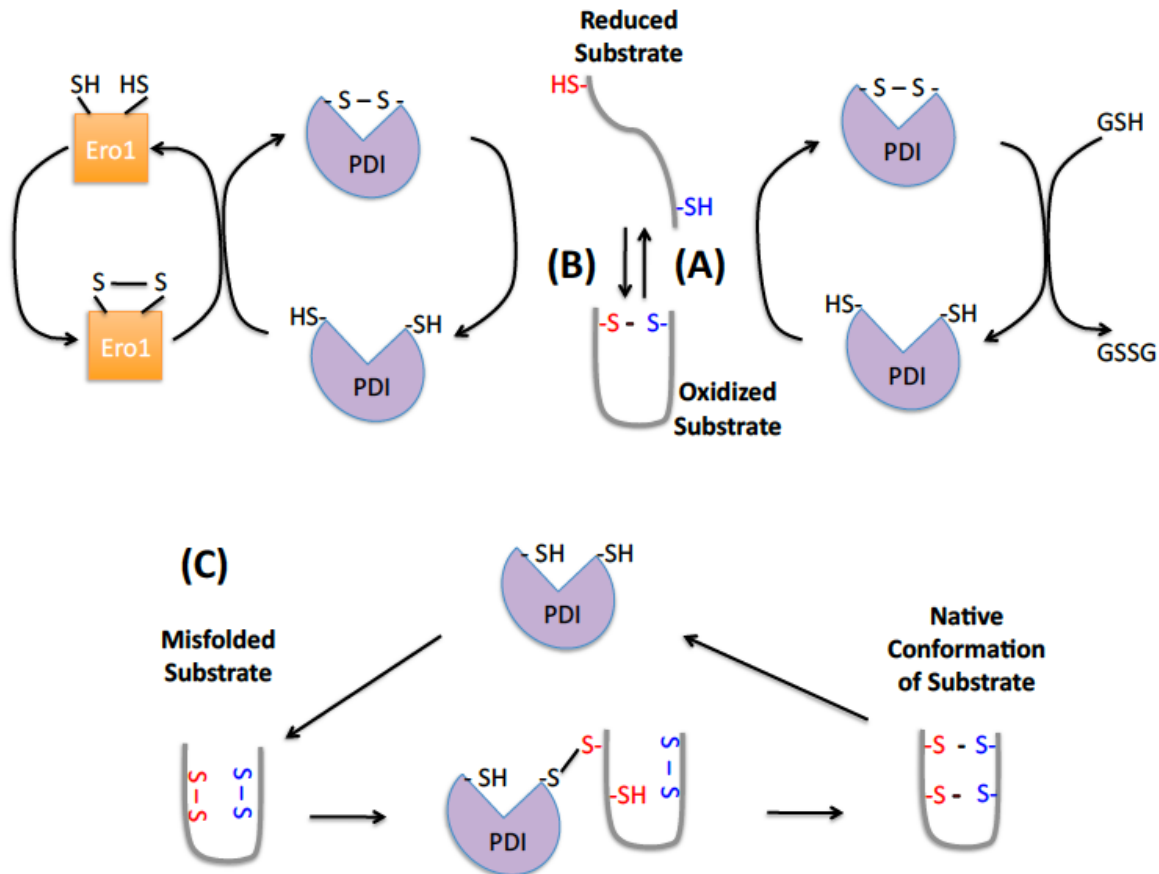


Figure 4. Role of PDIs in oxidation, reduction, and isomerization reactions

(A) Reduction of a substrate by PDI occurs when PDI in its reduced state donates electrons to the substrate, forming a disulfide bond between its own cysteines and breaking the disulfide bond of the substrate, forming two sulfhydryl groups. PDI in its oxidized state can be reduced by reduced glutathione (GSH) or Ero1. (B) Oxidation of a substrate occurs when electrons are transferred from the disulfide in oxidized PDI to the reduced substrate, forming a disulfide bond in the substrate and leaving PDI in the reduced state. PDI can be re-oxidized by Ero1 and GSSG. (C) During isomerization reactions there is no net change in redox potential, but substrates cycle forming intermediate mixed disulfides with PDI before achieving their native conformation.

1.3.3.2 Yeast PDIs

The physiological functions of PDI family members are not currently clearly defined. Members of the PDI family are identified by having sequence or structural homology to thioredoxin, but not all PDI family members have been shown to exhibit redox activity (Hatahet and Ruddock, 2009). Different organisms have different numbers and types of PDIs, some of which may be functionally related and others that appear to be unique to a subset of organisms. The yeast, *Saccharomyces cerevisiae*, has five PDI family members (Tachikawa *et al.*, 1991; Tachibana and Stevens, 1992; Tachikawa *et al.*, 1995; Tachikawa *et al.*, 1997; Wang and Chang, 1999; Xiao *et al.*, 2004; Hatahet and Ruddock, 2009), which are among the best characterized of this class of proteins. In contrast, humans have 21 currently identified PDI family members (Benham, 2012). See Table 1 for a list of all currently identified yeast and human PDIs.

Of the five genes encoding yeast PDI family members - *PDII*, *MPDI*, *MPD2*, *EUG1*, and *EPSI* - only one, *PDII*, is essential (Farquhar *et al.*, 1991). However, any of these genes can compensate for the loss of *PDII* when over-expressed (Norgaard *et al.*, 2001). Interestingly, when all five of the yeast PDI genes are deleted, only expression of *PDII* or over-expression of *MPDI* can restore viability (Norgaard *et al.*, 2001), implying that the yeast PDIs are not completely functionally redundant.

Yeast Pdi1 shares a similar domain organization to human PDI (a-b-b'-a'), having two active sites and two catalytically inactive sites, with the b' domain providing a hydrophobic chaperone-like binding pocket (Holst *et al.*, 1997; Gillece *et al.*, 1999; Tian *et al.*, 2008). The crystal structure of Pdi1 indicates that the protein forms a U-shaped molecule with the two redox active sites located across from each other at the top of the "U" and the substrate binding domain located within the base or pocket of the "U" opening. It is thought that substrates may bind in

the b' domain and the redox active arms of Pdi1 may bend down to interact with the substrate (Tian *et al.*, 2008).

The other four yeast PDIs share similarities with Pdi1, but also have unique attributes (Table 1) (Norgaard *et al.*, 2001). Mpd1 and Mpd2 are both soluble PDIs that have only one active site domain (a domain), with the sequences CGHC and CQHC respectively (Tachikawa *et al.*, 1995; Tachikawa *et al.*, 1997). Relatively little is known about Mpd2, but the crystal structure of Mpd1 was solved (Vitu *et al.*, 2008) and shows a protein with two clear thioredoxin-like folds, one redox active (a domain) and one redox inactive (b domain). The relative orientation of the two Mpd1 domains is quite different compared to Pdi1. While the active site domains of Pdi1 are thought to act as flexible arms, the two domains of Mpd1 are fixed and are inflexible (Vitu *et al.*, 2008). The differences in the crystal structures of the two yeast PDIs most likely indicate substrate specificity among these proteins. Another interesting feature of Mpd1 is that it associates with Cne1, the yeast homolog of calnexin, and this association increases the reductive potential of Mpd1 and abolishes the chaperone activity of Cne1 (Kimura *et al.*, 2005).

Eug1 is another soluble yeast PDI and shares the same domain orientation as Pdi1 (a-b-b'-a') (Tachibana and Stevens, 1992). Interestingly, the two active sites of Eug1 have a C-terminal serine residue instead of a cysteine residue, C-X-X-S. The a and a' active site sequences are CLHS and CIHS respectively (Tachibana and Stevens, 1992). These active site sequences indicate that Eug1 may have isomerase activity, but not oxidation or reduction potential, since these functions require thiols from two cysteine residues in one active site to cycle between an oxidized and reduced state to interact with substrates (Figure 4). Interestingly, although Eug1 does not have redox activity, it has been shown to interact with the last member of the yeast PDI family, Eps1, and to enhance its reductive and chaperone activities (Kimura *et al.*, 2005).

The final yeast PDI family member is Eps1, the only transmembrane protein among this group. Eps1 is homologous to the mammalian PDI TMX and has been shown to bind to Kar2, the yeast homolog of BiP, Pdi1, Mpd1, and Eug1 (Kimura *et al.*, 2005). Eps1 has one active site with the sequence CPHC (Wang and Chang, 1999). Eps1 has been implicated in ER quality control, as deletion of this gene suppresses a dominant negative mutation of the plasma membrane ATPase, Pma1-D378N (Wang and Chang, 1999). The plasma membrane localized Pma1 is essential for yeast growth and the D378N mutation prevents this protein from trafficking to the plasma membrane; however, upon deletion of *EPS1*, both wild type and mutant Pma1 traffic normally to the plasma membrane, indicating that Eps1 is involved in retaining the mutant protein in the ER. Eps1 may act to recognize the mutant form of Pma1 through its chaperone binding domain at the plasma membrane and act as a bridge to other PDI proteins or to Kar2 since it interacts with each of these. Interestingly, Eps1 does not affect other ERAD substrates, indicating that there is specificity among the family members.

1.3.3.3 Mammalian PDIs

Each of the 21 human PDIs (Table 1) shares at least one domain with similarity to one of the four PDI domains (Benham, 2012). Due to similarities in their domain organizations and active sites it is likely that at least a subset of PDIs will have similar redox potentials, although the kinetics of these reactions may differ. PDI, ERp57, PDIp, ERp72, and P5 when recombinantly expressed and purified all show similar ability *in vitro* to catalyze the oxidation of a disulfide bond in a peptide substrate (Alanen *et al.*, 2006). However, the large number, unique tissue distribution, and features of the PDIs may indicate distinct physiological functions for each of the family members. The physiological functions may be determined by their interactions with other proteins and not necessarily by their disulfide chemistry. Due to the sizeable number

of PDIs, detailed physiological studies of each of their individual functions have not been performed, but initial studies on select family members have been executed. Examples of physiological functions of some of the better characterized PDI family members follow. For a listing of known substrates of PDIs see Table 1.

Table 1. Properties and substrates of identified PDI family members

	Name	Domains	Active Site Sequences	Known Substrates*
Human PDI family members	PDI	a-b-b'-a'	CGHC, CGHC	Ero1 α , ApoB, cholera toxin, SV40, pro-insulin, lysozyme, ADAM17
	PDIP	a-b-b'-a'	CGHC, CTHC	
	ERp57	a-b-b'-a'	CGHC, CGHC	Ero1 α , SV40, MHC-I, laminin B3, collagen, clusterin, lysyl hydroxylase 2, laminins (B1 and γ 1), Integrins, LDL receptor, Plexin A1, Agrin, Fibrillin, Fibulin-like Protein, Discoidin, Lysyl oxidase 2, ADAM17, Adam10, Melanotransferin, Glucosyltransferase family member, Tapasin,
	ERp72	a ^o -a-b-b'-a'	CGHC, CGHC, CGHC	thrombospondin, thyroglobulin
	PDIR	b-a ^o -a-a'	CSMC, CGHC, CPHC	
	P5	a-a'-b	CGHC, CGHC	MICA
	PDILT	a-b-b'-a'	SKQS, SKKC	calmegin, calsperin
	ERdJ5	J-a-b-b-a-a-a	CSHC, CPPC, CHPC, CGPC	
	ERp44	a-b-b'	CRFS	adiponectin, IP3 Receptor
	ERp46	a ^o -a-a'	CGHC, CGHC, CGHC	Ero1 α , lysyl hydroxylase 2, laminins (B1, B2, B3, γ 1 and γ 2), Integrins, collagen, LDL receptor, ERAP1, ERAP2, EDEM3, LTBP1, Polycystic Kidney Disease 1, apartyl/arpargine B-hydroxylase, Nidogen-1, Peroxiredoxin IV
	ERp18	a	CGAC	Ero1 α , lysyl hydroxylase 2, laminins (B3 and γ 2), ER glucosidase II, PTX3
	ERp27	b-b'	none	
	ERp29	b'-D	none	polyomavirus VPI
	TMX	a	CPAC	
	TMX2	a	SNDC	
	TMX3	a-b-b'	CGHC	
	TMX4	a	CPSC	
	TMX5	a	CRFS	
	AGR2	a	CPHS	
	AGR3	a	CQYS	
	ERp90	Trx1-5	unknown	
Yeast PDI family members	Pdi1	a-b-b'-a'	CGHC, CGHC	p α f, CPY*
	Mpd1	a-b	CGHC	
	Mpd2	a	CQHC	
	Eug1	a-a'	CLHS, CIHS	
	Eps1	a	CPHC	Pma1-D378N

* from (Hatahet and Ruddock, 2009; Jessop et al., 2009; Benham 2012)

PDIs play an important role in lipid homeostasis. As described in section 1.2.2, PDI is a subunit of MTP that is responsible for loading lipids onto ApoB as it is translocated into the ER (Wetterau *et al.*, 1990). PDI is thought to retain the M-subunit of the complex in the ER, since it does not have an ER retention signal (Wetterau *et al.*, 1991). Interestingly, when PDI levels were reduced specifically in hepatocytes, MTP activity decreased by approximately 40%, consistent with the reduction in PDI (Wang *et al.*, 2012). A second PDI involved in lipid homeostasis is ERp44, which is involved in the quality control of adiponectin (Wang *et al.*, 2007) and regulating the Inositol Phosphate-3 Receptor Type I (Higo *et al.*, 2005).

PDIs have also been implicated in developmental regulation and cancer. In zebrafish, P5 has been shown to be required for asymmetric patterning of the heart, pancreas, liver, and gastrointestinal tract (Hoshijima *et al.*, 2002). When P5 is inactivated with morpholinos, the patterning defect can be rescued with exogenous expression of P5, indicating that the deficiency arises solely from the loss of P5 (Hoshijima *et al.*, 2002). P5 has also been linked to cancer. P5 associates with MICA, a ligand for the natural killer cell activating receptor on the surface of tumor cells and reduces its disulfide bond, facilitating a cleavage reaction which allows MICA to be shed from the cell surface (Kaiser *et al.*, 2007). MICA shedding aids the tumor cell in immune evasion (Kaiser *et al.*, 2007). In turn the PDI family member AGR2 is important for ER homeostasis and is upregulated in a number of cancer cells, such as malignant prostate epithelial cells (Fletcher *et al.*, 2003), ovarian high-grade serous carcinoma cells (Fletcher *et al.*, 2003; Darb-Esfahani *et al.*, 2012), and, along with another PDI family member, AGR3, in estrogen-positive breast tumors (Thompson and Weigel, 1998). Additionally, PDI is upregulated in several human cancers, although the relevance of upregulation is not clear. These include prostate (Welsh *et al.*, 2001), lung (Beer *et al.*, 2002; Basso *et al.*, 2005), and melanoma

(Talantov *et al.*, 2005; Lovat *et al.*, 2008). In contrast, PDI's activity is essential for survival and proliferation of ovarian cancer cells (Xu *et al.*, 2012). A recent study shows that inhibition of PDI using a small molecule inhibitor suppressed ovarian tumor growth without causing toxicity in non-cancerous cells (Xu *et al.*, 2012). Long term effects of blocking PDI function in normal cells was not addressed.

A final function for PDIs, and one that is most relevant to the work described in Chapter 2 is in ER quality control, through ERAD and ERAD-like phenomena. The PDI ERp57 interacts with ER lectins calnexin and calreticulin (Oliver *et al.*, 1999) and catalyzes the isomerization of disulfide bonds in glycoprotein substrates (Jessop *et al.*, 2009). If these substrates fail to properly fold, they are selected for ERAD. For proteins to be degraded by ERAD they must be retrotranslocated from the ER into the cytosol, but in order for retrotranslocation to take place, disulfide bonds may have to be reduced. Notably, the PDI ERdj5 reduces the disulfide bonds of misfolded proteins, and in association with EDEM and the chaperone BiP, promotes their ERAD (Ushioda *et al.*, 2008; Hagiwara *et al.*, 2011). In addition, viruses and bacteria have evolved to co-opt the ERAD machinery, allowing them exit from the ER to the cytosol for productive infection. *Vibrio cholerae* secrete cholera holotoxin, which is disassembled in the ER by PDI, facilitating the retrotranslocation of cholera toxin into the cytosol (Tsai and Rapoport, 2002; Taylor *et al.*, 2011). Furthermore, ERp29 triggers a conformational change in the polyomavirus protein VP1, which is necessary for its export into the cytosol for viral infection (Magnuson *et al.*, 2005; Rainey-Barger *et al.*, 2009). Another polyomavirus, SV40, requires PDI and ERp57 for its transport from the ER lumen (Schelhaas *et al.*, 2007).

At the start of my thesis project, very few PDI substrates had been identified and a significant question in the field was whether PDIs exhibit substrate specificity. As time

progresses, more substrates and physiological roles for mammalian PDIs are being uncovered and it is becoming more obvious that there is specificity among the various members of this family; however, we are far from identifying all of the substrates and actions of the PDIs. A primary hypothesis underlying my studies has been that ApoB is a potential substrate for PDI family members. ApoB has 25 cysteines and forms eight disulfide bonds (Yang *et al.*, 1989) that are necessary for the correct assembly and secretion of VLDL (Huang and Shelness, 1997; Tran *et al.*, 1998; DeLozier *et al.*, 2001). ApoB has been shown to interact with PDI through the MTP complex (Hussain *et al.*, 2003) and to associate with two other PDIs, ERp57 and ERp72 (Adeli *et al.*, 1997; Linnik and Herscovitz, 1998; Zhang and Herscovitz, 2003). The function of these PDI family members in ApoB biogenesis however, had not yet been addressed. In Chapter 2, I investigated the role of PDI family members in the degradation and secretion of ApoB.

1.4 APOLIPOPROTEIN B REGULATION AND RELATED DISEASES

ApoB transport is essential for maintaining cellular homeostasis, and as a result defects in ApoB synthesis or regulation can lead to a number of diseases. Naturally occurring non-sense or frameshift mutations in the ApoB gene result in the production of truncated versions of the ApoB protein; the truncated proteins are less competent to carry lipids than ApoB100 and result in low circulating levels of VLDL (McLeod *et al.*, 1994; Whitfield *et al.*, 2004). Patients with these mutations have a condition known as hypobetalipoproteinemia, and exhibit symptoms that are associated with fat malabsorption such as ataxia, neuromuscular degeneration, and fat-soluble vitamin deficiencies (Linton *et al.*, 1993; Whitfield *et al.*, 2004). The symptoms result from the

fact that peripheral tissues in these patients are starved for sufficient cholesterol and fatty acids. Additionally, patients develop a fatty liver because the tissue becomes overloaded with endogenously synthesized cholesterol that is unable to be secreted (Linton *et al.*, 1993; Whitfield *et al.*, 2004). A second rare autosomal recessive disorder that results in the complete absence of ApoB containing lipoproteins is called abetalipoproteinemia. Abetalipoproteinemia results from mutations that ablate the function of the MTP complex (Wetterau *et al.*, 1992), which is necessary for loading ApoB with lipids (see section 1.2.2). Patients with abetalipoproteinemia develop multi-systemic symptoms and are unable to solubilize fats and fat-soluble vitamins, which leads to failure to thrive and vitamin deficiencies (Whitfield *et al.*, 2004). Interestingly, a long-term, high-dose oral vitamin E treatment has been shown to eliminate some of the clinical manifestations of abetalipoproteinemia (Zamel *et al.*, 2008).

In addition to human diseases arising from decreased circulating ApoB-containing lipoproteins, high circulating levels of ApoB have been linked to the development of atherosclerotic plaques and subsequently coronary artery disease (Schayek and Eisenberg, 1994; Ginsberg, 2002). In this much more common ApoB-linked disease, elevated levels of circulating LDL increase the risk of the lipoprotein being absorbed into the endothelial lining of the blood vessel wall where it can become oxidized (Kume *et al.*, 1992; Camejo *et al.*, 1998; Skalen *et al.*, 2002; Leitinger, 2003; Benn, 2009). Oxidized LDL particles in the arterial wall trigger an inflammatory response, which generate an atherosclerotic lesion. The lesions thicken the arterial wall and decrease the diameter of the artery, thus reducing the amount of blood that can pass through (Leitinger, 2003; Olofsson and Boren, 2005; Benn, 2009). The resulting atherosclerotic plaques increase the risk of having a blood clot, heart attack, and stroke.

Coronary artery disease results from the hardening and narrowing of arteries due to the accumulation of atherosclerotic plaques that obstruct blood flow to the heart and is a leading cause of death in the United States. Many cases of familial coronary artery disease have been linked to mutations in the LDLR that decrease the affinity of the receptor for ApoB (Goldstein and Brown, 1982). As a result, more LDL particles circulate in the bloodstream and are unable to be cleared. Several other factors can also contribute to high serum levels of LDL; for instance, a diet rich in fat, mutations in the TAG metabolizing enzyme LPL, and increased expression of ApoC-III, which inhibits LPL, can all lead to elevated levels of LDL in the bloodstream and atherosclerosis (Kwiterovich, 2000).

Treatments for reducing plasma cholesterol carried by ApoB-containing lipoproteins are currently focused on statin treatment; there are several other medications that can lower LDL levels, albeit to a lesser extent (Joy, 2012). Statins are a class of cholesterol lowering drugs that act by decreasing the synthesis of cholesterol in the liver by inhibiting the enzyme 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase (see section 1.4.2) (Endo *et al.*, 1976). Unfortunately, up to 25% of patients using statins are unresponsive to the treatment or have serious side effects such as myopathy and kidney disease (Yan *et al.*, 2006). Another serum cholesterol lowering treatment in clinical trials is mipomersen, an antisense oligonucleotide that inhibits expression of the APOB gene, thus reducing the amount of cholesterol that can be packaged and transported through the bloodstream (Ito, 2007). Trials with mipomersen have reported modest decreases in serum cholesterol levels, although adverse side effects such as flu-like symptoms and increased intra-hepatic TAG levels were reported (Kastelein *et al.*, 2006; Akdim *et al.*, 2010; Visser *et al.*, 2010; Akdim *et al.*, 2011). A third group of cholesterol lowering treatments are MTP inhibitors (Joy, 2012). As evident by patients with abetalipoproteinemia, inhibiting MTP prevents ApoB-

containing lipoproteins from circulating in the bloodstream. Non-specific as well as intestine-specific MTP inhibitors are in development, but clinical trials for some of these drugs have been stalled due to increases in hepatic TAG levels (Chandler *et al.*, 2003). However, one non-specific MTP inhibitor, Lomitapide, reduced TAG levels by 87% and LDL levels by 29% in rats (Dhote *et al.*, 2011), but in human trials, adverse gastrointestinal and liver effects were observed (Cuchel *et al.*, 2007).

Currently each of the possible treatments for high serum cholesterol levels is either not sufficiently effective or comes with the risk of serious side effects (Joy, 2012). Therefore, reducing plasma levels of ApoB through other methods will be greatly aided by identifying new therapeutic targets. In order to identify new targets for reducing serum cholesterol levels, I have focused my efforts on understanding how ApoB is regulated.

1.4.1 Regulation of Apolipoprotein B Synthesis

As mentioned in section 1.2.1 ApoB48 transcription is regulated by the enzyme apobec-1. In humans, apobec-1 is expressed specifically in enterocytes and converts a cytidine in the ApoB mRNA to a uridine, creating a premature stop codon that creates a truncated version of the protein (Chen *et al.*, 1987; Navaratnam *et al.*, 1993). As ApoB48 is found only in chylomicrons, which absorb dietary cholesterol, this conversion is restricted to the small intestine.

Surprisingly, ApoB levels are predominantly regulated by degradation and not by the amount of ApoB synthesis; however, the availability of cholesterol and lipids determines whether ApoB is secreted or degraded and whether synthesis in the cholesterol biosynthetic pathway is regulated. A rate-limiting step in the mevalonate pathway that generates cholesterol

and other isoprenoids is the conversion of HMG-CoA to mevalonate by the enzyme HMG-CoA reductase (Goldstein and Brown, 1990), this enzyme is the target of the statins. HMG-CoA reductase transcription is regulated by sterol-mediated feedback inhibition, ensuring that cholesterol synthesis meets but does not exceed cellular requirements (Siperstein and Guest, 1960; Goldstein and Brown, 1990; Goldstein *et al.*, 2006). When cholesterol is depleted, the transcription factor sterol regulatory element-binding protein (SREBP) traffics to the Golgi where it is cleaved and activated, allowing it to enter the nucleus and activate HMC-CoA reductase transcription (Espenshade and Hughes, 2007). Additionally, HMG-CoA reductase can be regulated by degradation via the ubiquitin-proteasome pathway, i.e. via ERAD (McGee *et al.*, 1996; Ravid *et al.*, 2000).

1.4.2 Regulation by Degradation

As mentioned in the preceding sections, the correct folding of ApoB requires its proper targeting to the ER, the addition of posttranslational modifications such as N-glycans and disulfide bonds, and lipid loading by the MTP complex. As evident by the fact that reduced levels of ApoB in the serum as well as excess circulating ApoB both lead to disease states, the assembly and secretion of ApoB-containing lipoproteins must be a highly regulated process. The regulation of ApoB occurs predominantly through cotranslational and posttranslational targeting of ApoB for degradative pathways (Brodsky and Fisher, 2008; Ginsberg and Fisher, 2009). These processes, described in the following sections, are mainly controlled by the availability of core lipids to load onto the lipoprotein, the variety of dietary fatty acids consumed, and by hormonal signaling.

1.4.2.1 Autophagy

One degradative pathway that regulates ApoB secretion is a form of autophagy called Post-ER Presecretory Proteolysis (PERPP) (Fisher *et al.*, 2001). Autophagy mediates the degradation of cytosolic proteins, protein aggregates, and fragments of damaged organelles, by engulfing the substrates in vesicles called autophagosomes so that they are delivered to the lysosome and degraded (Levine and Kroemer, 2008; Martinez-Borra and Lopez-Larrea, 2012). PERPP is a posttranslational process that acts on ApoB that has been lipid loaded and trafficked from the ER to the Golgi but that has been damaged by oxidative stress, causing the formation of aggregates (Fisher *et al.*, 2001; Djousse *et al.*, 2003). Polyunsaturated fatty acids consumed in the diet, such as eicosapentaenoic acid and docosahexaenoic acid, stimulate PERPP due to the oxidative stress caused by their metabolism (Pan *et al.*, 2004). Interestingly, these data correlate with studies indicating that a diet rich in omega-3 fatty acids, which oxidize ApoB to result in a decreased risk for the development of coronary artery disease (Williams and Fisher, 2005). The oxidative stress-induced degradation of ApoB aggregates requires active autophagy since this process is inhibited by the compounds 3-methyladenine and E64d, or when the autophagy-requiring gene Atg7 is depleted by siRNA treatment (Pan *et al.*, 2008).

In addition to PERPP, which is stimulated by polyunsaturated fatty acids, there are other examples of post-ER degradation of ApoB. The ApoB mutant A31P that is associated with familial hypobetalipoproteinemia was found to traffic through the ER and to the Golgi; however, secretion of this mutant protein was impaired (Zhong *et al.*, 2010). It was discovered that the A31P mutant was degraded by the lysosome after trafficking to the Golgi (Zhong *et al.*, 2010). In addition to polyunsaturated fatty acids, another factor that enhances the degradation of ApoB

by PERPP is insulin (Chirieac *et al.*, 2000; Taghibiglou *et al.*, 2000; Chirieac *et al.*, 2004; Biddinger *et al.*, 2008). While the mechanisms that select ApoB for PERPP are still poorly understood, the Golgi-localized protein sortilin was recently shown to act as a sorting receptor that binds to ApoB-containing lipoproteins and targets them for lysosomal degradation (Musunuru *et al.*, 2010; Strong *et al.*, 2012). Future research efforts are sure focus on defining the factors that specifically target ApoB to the autophagic pathway and to the lysosome, and may uncover new therapeutic targets for lowering the production of atherogenic lipoproteins.

1.4.2.2 Endoplasmic Reticulum Associated Degradation (ERAD)

While degradation by PERPP occurs posttranslationally and is dependent on lysosomal proteases, the second degradative pathway that regulates ApoB biogenesis is ERAD and occurs cotranslationally. ERAD is a quality control mechanism that prevents the accumulation of misfolded proteins in the ER through molecular chaperone recognition, polyubiquitination, retrotranslocation, and proteasomal degradation of the misfolded substrate (for a recent review see (Guerriero and Brodsky, 2012)). Interestingly, the ERAD of ApoB is unique because it occurs cotranslationally and is metabolically regulated (Dixon *et al.*, 1991; Yeung *et al.*, 1996; Benoist and Grand-Perret, 1997; Fisher *et al.*, 1997; Yao *et al.*, 1997; Liao *et al.*, 1998; Mitchell *et al.*, 1998; Gusarova *et al.*, 2001; Pariyarath *et al.*, 2001; Liang *et al.*, 2003; Oyadomari *et al.*, 2006; Fisher *et al.*, 2008).

As ApoB is synthesized by the ribosome, it is cotranslationally translocated into the ER, where it is loaded with lipids by the MTP complex (see above and section 1.2 (Hussain *et al.*, 1997)). When the MTP complex is functional and lipids are abundant, ApoB is fully translocated into the ER and assembles into a pre-VLDL that can be trafficked from the ER to

the Golgi, where it undergoes further maturation (Figure 5)(Gusarova *et al.*, 2003). Within the ER lumen, ApoB interacts with a number of molecular chaperones that help fold ApoB into its correct conformation and determine if it has properly assembled into a pre-VLDL. Some of these chaperones include BiP, p58^{IPK}, GRP94, ERp57, ERp72, PDI, calnexin, and calreticulin (Adeli *et al.*, 1997; Benoist and Grand-Perret, 1997; Linnik and Herscovitz, 1998; Tatu and Helenius, 1999; Zhang and Herscovitz, 2003; Oyadomari *et al.*, 2006). When ApoB is improperly folded, these chaperones target the protein for ERAD, although in most cases this has not been specifically shown. In one example however, glucosamine treatment of hepatic cells led to increased levels of BiP in the ER and, in turn, ApoB100 ubiquitination and proteasomal degradation were enhanced (Qiu *et al.*, 2005). Additionally, in the absence of the BiP interacting co-chaperone p58^{IPK} ApoB ERAD was reduced (Oyadomari *et al.*, 2006).

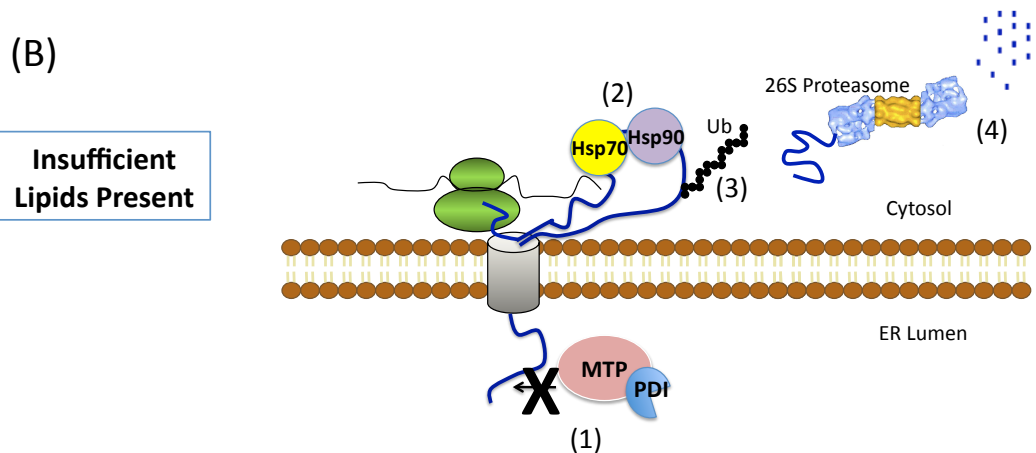
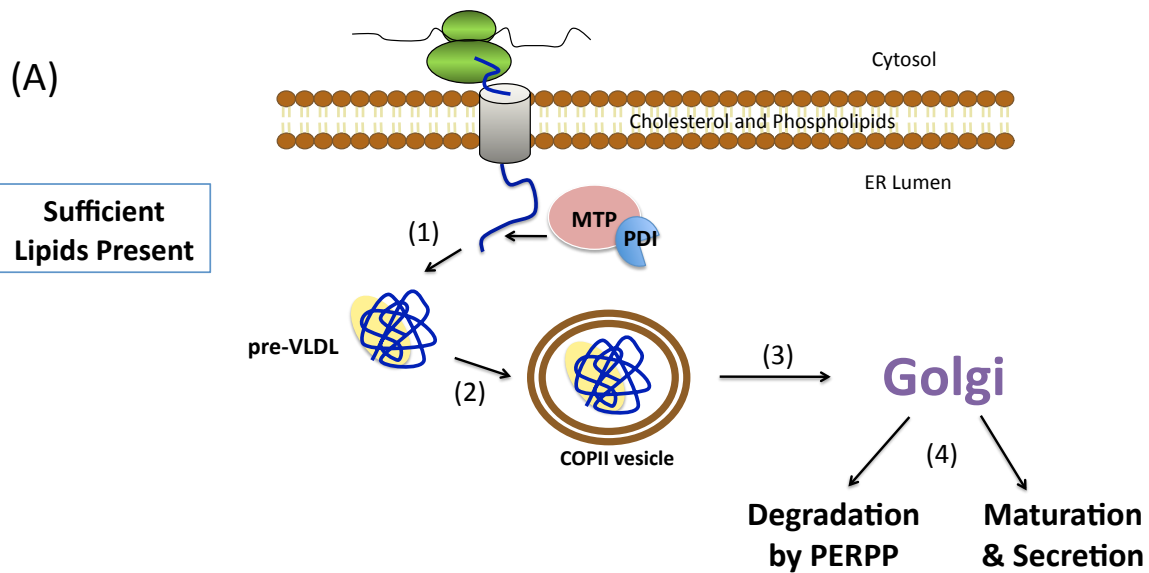


Figure 5. ApoB ERAD is metabolically regulated by lipid availability

(A) (1) When a sufficient amount of lipids are available in the ER, the MTP complex loads ApoB with lipids and it is assembled into a pre-VLDL. (2) The pre-VLDL is packaged into a COPII vesicle and (3) traffics from the ER to the Golgi. (4) If the pre-VLDL passes a quality control event it undergoes further maturation and can be secreted. If the lipoprotein has been oxidatively damaged or does not pass quality control, it is degraded by PERPP. (B) (1) When lipids are limiting in the ER, the MTP complex cannot assemble ApoB into a pre-VLDL. (2) ApoB loops accumulate in the cytosol due to ongoing synthesis that are recognized by the heat shock proteins Hsp70 and Hsp90. (3) ApoB is polyubiquitinated, retrotranslocated out of the ER and into the cytosol and (4) degraded by the 26S proteasome.

When the MTP complex is not fully functional, or during times when the intracellular concentrations of phospholipids and TAG are depleted, cotranslational translocation of ApoB into the ER stalls and loops of ApoB protein are exposed to the cytosol due to continued translation in the absence of translocation (Dixon *et al.*, 1991; Yeung *et al.*, 1996; Fisher *et al.*, 1997; Liao *et al.*, 1998; Pariyarath *et al.*, 2001; Cardozo *et al.*, 2002). The cytoplasmic loops of ApoB are bound by the heat shock proteins Hsp70 and Hsp90, which target the protein for degradation by ERAD (Fisher *et al.*, 1997; Gusarova *et al.*, 2001). Following recognition by the heat shock proteins, ApoB is poly-ubiquitinated by the E3 ubiquitin ligase Gp78, retrotranslocated out of the same Sec61 translocon into which it was inserted, and degraded by the 26S proteasome (Figure 5) (Yeung *et al.*, 1996; Benoist and Grand-Perret, 1997; Zhou *et al.*, 1998; Du *et al.*, 1999; Gusarova *et al.*, 2001; Zhang *et al.*, 2001; Liang *et al.*, 2003). Unlike the majority of ERAD substrates that are fully translated and translocated into the ER lumen or membrane before they are targeted for ERAD, ApoB is unique in that it is degraded cotranslationally and remains associated with the Sec61 translocon during degradation (Mitchell *et al.*, 1998; Oyadomari *et al.*, 2006).

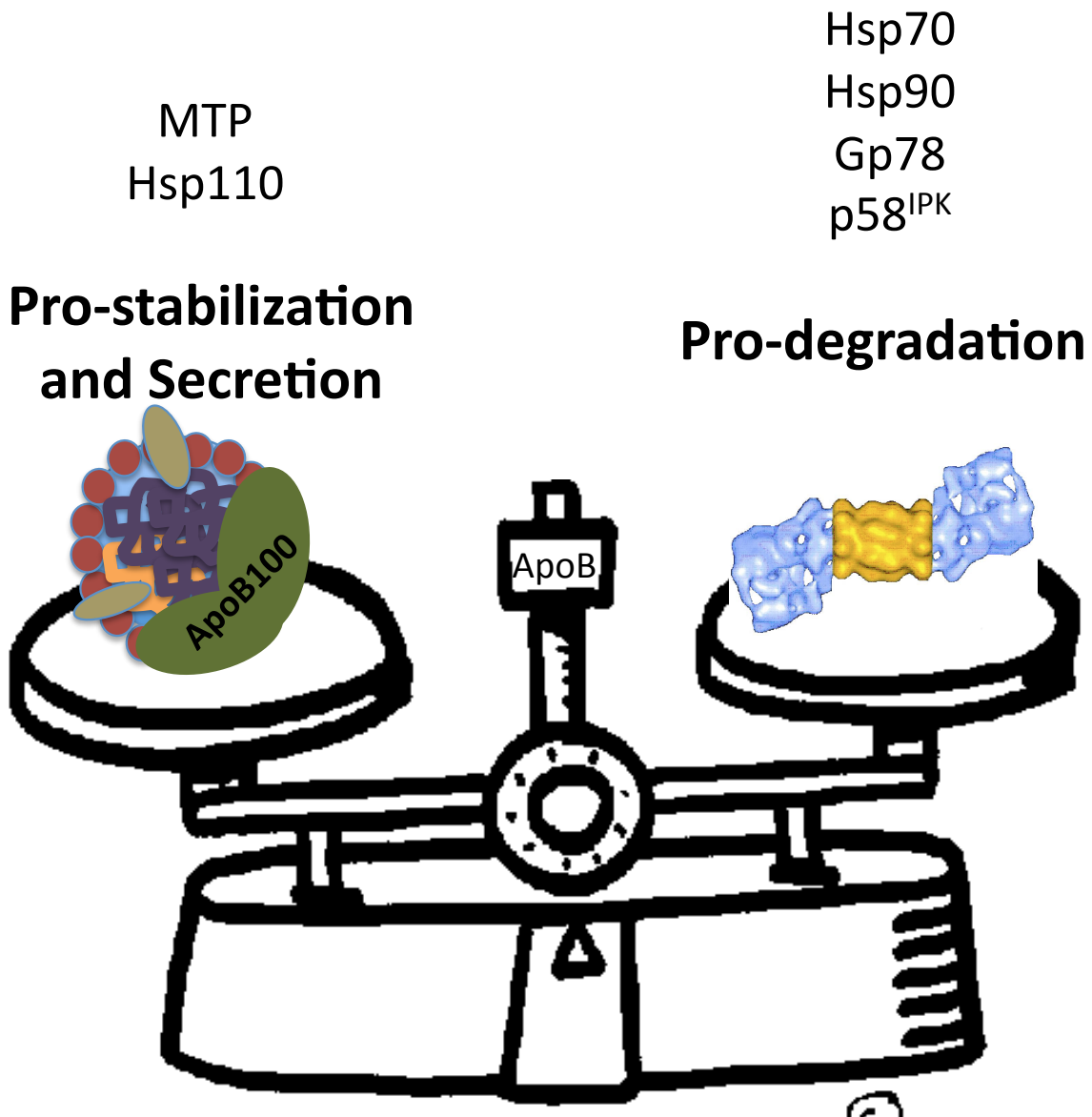


Figure 6. Mammalian proteins that contribute to ApoB biogenesis

The secretion and degradation of ApoB are finely balanced to ensure that the risk of disease from inadequate or excess circulating cholesterol is minimized. The MTP complex and the heat shock protein Hsp110 have been shown to favor ApoB stabilization and promote its secretion, while p58^{IPK}, Gp78, and the heat shock proteins Hsp70 and Hsp90 have been shown to promote ApoB degradation when conditions do not favor lipoprotein assembly.

Although a number of factors involved in the ERAD of ApoB have been identified (see below) and (Figure 6), it is likely that many more contribute to this process, and several questions with regards to the mechanism underlying ApoB degradation remain. For example, Gp78 has been identified as the E3 ubiquitin ligase for ApoB (Liang *et al.*, 2003), but there are many functionally redundant E3s in the cell (Nakatsukasa *et al.*, 2008). Do any of the other ubiquitin ligases participate in ApoB turnover? Additionally, a previous graduate student in the Brodsky lab, Stacy Hrizo showed that ApoB29 is stabilized in yeast lacking the E2 ubiquitin conjugating enzyme Ubc7; however, the mammalian E2 for ApoB has not been identified. As mentioned above and in section 1.3.3 ApoB forms eight disulfide bonds and interacts with the PDI family members PDI, ERp57, and ERp72. Although PDIs have been shown to function in ERAD and ERAD-like processes (Wang and Chang, 2003; Schelhaas *et al.*, 2007; Ushioda *et al.*, 2008; Moore *et al.*, 2010; Hagiwara *et al.*, 2011), and that ERp57 and ERp72 bind ApoB it is unknown whether these PDIs directly contribute to ApoB degradation, which was a main focus of my dissertation research. In addition, whether the ER mannosidase, EDEM-I, which interacts with proteins and targets them for degradation, is involved in the recognition and degradation of ApoB has not been investigated. Another important question regards the fact that the ribosome associates with the Sec61 translocon pore to facilitate entry of proteins into the ER. But, during ApoB retrotranslocation this association must change, allowing a gap to form between the ribosome and Sec61 so that ApoB loops can slip out into the cytosol. Are there proteins that regulate the interaction between the ribosome and Sec61 or facilitate the reversed direction of ApoB transport through the channel, an event that dictates translocation vs retrotranslocation? Finally, after ApoB is retrotranslocated from the ER, how is the lipoprotein maintained in a soluble state in the cytosol before being degraded by the proteasome? Are there chaperones or

chaperone-complexes that prevent ApoB aggregation in the cytosol? Some of these questions have been addressed in Chapters 2 and 3, while speculation as to the answers of the other questions are presented in Chapter 4.

1.5 MODEL SYSTEMS TO STUDY APOB

1.5.1 Mouse Models

When studying atherosclerosis in animals, the mouse model is the predominant system, due to the ability to construct transgenics and the significant decrease in cost compared to rabbits and primates (Veniant *et al.*, 2008). A number of knockout and conditional mice have been created to study lipoprotein trafficking, with the hope that we can better understand the processes leading to the development of atherosclerosis. LDL receptor deficient mice ($Ldlr^{-/-}$) have been used to model familial hypercholesterolemia (Ishibashi *et al.*, 1993; Ishibashi *et al.*, 1994; Farese *et al.*, 1996; Powell-Braxton *et al.*, 1998). When fed on a normal chow diet, these mice develop moderate increases in circulating LDL associated cholesterol and atherosclerotic lesions (Nakashima *et al.*, 1994). Additionally, to better study the contribution of ApoB isoforms to the development of atherosclerosis, the Young lab developed models with mice that solely expressed ApoB48 ($Apob^{48/48}$) or ApoB100 ($Apob^{100/100}$) (Veniant *et al.*, 1998). When the $Apob^{100/100}$ mice were crossed with $Ldlr^{-/-}$ mice the offspring had extremely high plasma cholesterol levels, much higher than the offspring when the same cross was performed with $Apob^{48/48}$ mice (Veniant *et al.*, 1998). These data provide support that the LDL receptor clears ApoB100 from the bloodstream. Another mouse model that contributed to our knowledge of the ApoB biogenesis

pathway was the MTP^{+/-} mouse. Consistent with MTP's role in the loading of lipids onto ApoB and the assembly of VLDLs, MTP^{+/-} mice had a 50% reduction in LDL cholesterol and a 28% reduction in circulating ApoB100 (Raabe *et al.*, 1998). In contrast, MTP^{-/-} mice were embryonically lethal (Raabe *et al.*, 1998), indicating the importance of lipid homeostasis in development.

Although the mouse model is extremely useful in understanding the full range of effects when ApoB biogenesis is modulated in an organism, teasing out pathways contributing to ApoB biogenesis and identifying key protein regulators can be quite difficult and time consuming. As a result, other model systems have been used to elucidate factors contributing to ApoB secretion and degradation, including primates, cell culture models, a cell-free *in vitro* system, and a yeast expression system.

1.5.2 Non-human Primates

Another organism used to study the contribution of ApoB to the development of atherosclerosis is a non-human primate, the cynomolgus monkey. To determine whether reduced levels of circulating ApoB could reduce serum cholesterol levels in primates, *APOB*-specific siRNAs were encapsulated in stable nucleic acid lipid particles and intravenously injected into monkeys (Zimmermann *et al.*, 2006). A dose dependent silencing of *APOB* was observed in the liver of treated monkeys and was maintained for 11 days following injection. As early as 12 hours after siRNA injection, plasma levels of ApoB100 were reduced and maximum reduction was achieved by 72 hours. Serum cholesterol levels were reduced by 62%, which in patients with hypercholesterolemia would be clinically significant and exceeds the amount of cholesterol reduction achieved by approved cholesterol lowering drugs (Zimmermann *et al.*, 2006). This

study was the first to show that clinically relevant RNAi treatments could be used in primates and supports the use of RNAi therapeutics as a new class of drugs. Additionally, this work established the cynomolgus monkey as a model for studying cholesterol related diseases in non-human primates.

1.5.3 Tissue Culture Systems

Three cell lines are most commonly used to study ApoB biogenesis, McArdle-RH7777 cells, Huh7 cells, and HepG2 cells. McArdle cells are rat hepatoma cells that express substantial amounts of both ApoB100 and ApoB48 (see section 1.2.1) (Fainaru *et al.*, 1977; Windmueller and Spaeth, 1985; Leighton *et al.*, 1990). McArdle cells express the MTP complex (Swift *et al.*, 2003), and as a result ApoB is loaded with lipids and secreted (Boren *et al.*, 1994). The Olofsson lab showed that in McArdle cells ApoB100 is secreted in lipoproteins corresponding to the density of VLDLs, and if the cells are supplemented with oleic acid, ApoB100 is also present in LDL particles (Boren *et al.*, 1994); however, ApoB48 was only secreted in VLDL particles in the presence of oleic acid (Boren *et al.*, 1994). These studies indicated that ApoB is synthesized and trafficked normally in McArdle cells, establishing this cell line as a good model system for studying ApoB biogenesis. Additionally, the Fisher lab has shown that polyunsaturated fatty acids in fish oils decrease ApoB secretion and increase intracellular degradation in McArdle cells (Wang *et al.*, 1993; Wang *et al.*, 1994).

While McArdle cells secrete much of their ApoB in lipoprotein particles, making them a favorable model system, it is often desirable to use a similar system of human origin. The other common and established cell culture systems for studying ApoB are HepG2 cells or Huh7 cells,

both of which are human hepatoma cell lines. Although ApoB is secreted predominantly as an LDL particle in these cells, nevertheless, in a normal human liver the majority of ApoB is secreted as a VLDL (Meex *et al.*, 2011). ApoB degradation is robust in these cells and the ubiquitin proteasome pathway has been shown to mediate ApoB turnover in this system (Brodsky and Fisher, 2008; Meex *et al.*, 2011). Together, HepG2 and Huh7 cells behave very similarly with respect to ApoB biogenesis and as such both are favorable models for studying ApoB.

1.5.4 Cell-free *In vitro* System

To investigate how ApoB interacts with select molecular chaperones and how ApoB is targeted for degradation, the Fisher lab developed a cell-free *in vitro* system to study the degradation of ApoB (Gusarova *et al.*, 2001). This system utilizes human ApoB48 that is *in vitro* transcribed and translated in the presence of dog pancreatic microsomes. The ApoB48 isoform was used for simplicity due to its smaller size and similar properties to ApoB100 in cell culture, with respect to its translocation, membrane integration, and degradation pathway (Davis *et al.*, 1990). The activity of the MTP complex and active lipid synthesis are absent in the cell-free system, making this a good model for the lipid deficient state in hepatic cells. In this system, the Fisher lab showed that ApoB48 is incorporated into the microsomes, is glycosylated, associates with microsomal membrane lipids, and is ubiquitinated. These properties demonstrated that the early events during ApoB biogenesis can be recapitulated *in vitro* and suggested that this system could be used to study factors that impact ApoB degradation. Using the *in vitro* system, Hsp90 was identified as a pro-degradation factor for ApoB, which was confirmed in rat hepatoma cells (section 1.5.2) (Gusarova *et al.*, 2001). Additionally, Sse1, the yeast homolog of Hsp110, was

identified as a pro-stabilization factor for ApoB using this system, which was also confirmed using the yeast expression system (section 1.5.5) and hepatic cells (section 1.5.3) (Hrizo *et al.*, 2007).

1.5.5 Yeast Expression System

In order to create a genetic system in which components required for ApoB biogenesis could be more rapidly screened and examined, the Brodsky lab developed a yeast expression system for the ApoB29 isoform (Hrizo *et al.*, 2007). As mentioned in section 1.2.1, ApoB29 is the shortest isoform of ApoB that traffics normally through the secretory pathway (Segrest *et al.*, 2001). Importantly, ERAD is a highly conserved process, and many yeast strains with deletions or mutations in the ERAD machinery are readily available. In this model system, ApoB29 expression was driven by a galactose inducible promoter and an HA-tag was appended. The signal sequence of ApoB was also replaced with a signal sequence from a yeast protein, pre-pro alpha factor, which had been previously used to direct the β -amyloid precursor protein to the yeast ER (Zhang *et al.*, 1994). This was necessary because the endogenous ApoB signal sequence exhibited poor yeast codon bias (Hrizo *et al.*, 2007). To validate the expression system, Hrizo *et al.*, showed that a protein of the expected molecular mass, that was recognized by both HA and anti-ApoB antibodies, was expressed in yeast and that ApoB29 localized to ER membranes in yeast and was associated with the Sec61 translocon. Additionally, ApoB29 was degraded in an ERAD dependant manner (Hrizo *et al.*, 2007), as would be expected since yeast lack the MTP complex. Using this expression system, the Brodsky lab then showed that Hsp110 contributes to the stabilization of ApoB, which was confirmed using hepatic cells (see above),

thus validating the use of this system to identify factors that regulate ApoB biogenesis. The majority of the work presented in this thesis was performed using the yeast expression system.

1.6 SUMMARY

Since the amount of secreted ApoB that circulates in the bloodstream correlates directly to serum cholesterol levels (Crooke *et al.*, 2005; Zimmermann *et al.*, 2006), a better understanding of ApoB secretion is vital for the treatment and prevention of hypobetalipoproteinemia and coronary artery disease. As described in section 1.4, ApoB secretion is tightly controlled, in large part through regulated degradation, an important component of which is accomplished via the ERAD pathway (Fisher and Ginsberg, 2002; Brodsky and Fisher, 2008; Rutledge *et al.*, 2010). ERAD involves four major steps: selection of a misfolded protein, retrotranslocation of the protein from the ER to the cytosol, substrate ubiquitination, and proteasome-mediated degradation, described section 1.4.2.2 (Meusser *et al.*, 2005; Vembar and Brodsky, 2008; Xie and Ng, 2010). Since ERAD results in lower intracellular and secreted levels of ApoB, it follows that a decline in ApoB production via ERAD will decrease serum lipid and cholesterol levels. Thus, I have sought to better define the ERAD pathway for ApoB using both yeast and mammalian cell culture systems, in an effort to provide novel future therapeutic targets to treat cardiovascular and other, related diseases.

In the following chapters I describe the identification of two classes of proteins that, through different mechanisms, impact ApoB secretion and/or degradation. I first asked whether members of the PDI family exhibit substrate specificity and contribute to the biogenesis of ApoB. In Chapter 2, I show that two members of the mammalian PDI family, ERp57 and

ERp72, promote ApoB ERAD, while another member, PDI, promotes ApoB secretion. I next focused on identifying regulators of the Sec61 translocon and asked whether selected candidate proteins are modulators of ApoB ERAD. In Chapter 3, I identify two members of the yeast Yet family of proteins, Yet2 and Yet3, as potential regulators of Sec61 that facilitate the ERAD of ApoB.

2.0 PROTEIN DISULFIDE ISOMERASES CONTRIBUTE TO THE ENDOPLASMIC RETICULUM-ASSOCIATED DEGRADATION OF APOLIPOPROTEIN B

Disulfide bonds, such as those found in ApoB, are formed within the oxidizing environment of the ER by PDI family members (Kleizen and Braakman, 2004; Anelli and Sitia, 2008; Hatahet and Ruddock, 2009; Brodsky and Skach, 2011; Bulleid and Ellgaard, 2011). PDIs are identified by the presence of one or more thioredoxin-like motifs (Cys-X-X-Cys) and can catalyze the oxidation, reduction, and/or isomerization of disulfide bonds. Along with catalytic redox or isomerase activity, some PDI family members also possess chaperone activity due to the presence of a hydrophobic substrate binding domain (Cai *et al.*, 1994; Song and Wang, 1995; Klappa *et al.*, 1998; Gillece *et al.*, 1999; Ellgaard and Ruddock, 2005; Tian *et al.*, 2006; Tian *et al.*, 2008; Hatahet and Ruddock, 2009). As noted in section 1.3.3, in mammals there are twenty-one PDI family members, one of which is known as PDI and associates with the M subunit to form the MTP complex; however, with few exceptions (Hatahet and Ruddock, 2009; Jessop *et al.*, 2009; Park *et al.*, 2009; Rutkevich *et al.*, 2010; Brodsky and Skach, 2011; Benham, 2012), specific functions for each of these proteins have not been assigned. In contrast, in the yeast *S. cerevisiae* there are five PDI family members, which are encoded by the *PDII*, *MPD1*, *MPD2*, *EUG1*, and *EPSI* genes. *PDII* is the only essential gene among this group (Farquhar *et al.*, 1991), and contains two active site thioredoxin-like domains (denoted the a and a' active sites),

each with a CXXC motif and a substrate binding domain in the b' domain (Holst *et al.*, 1997; Gillece *et al.*, 1999; Tian *et al.*, 2008). Although *PDII* is essential, the expression of Mpd1 from the *PDII* promoter supports cell viability in *pdil* Δ yeast (Norgaard *et al.*, 2001).

Select examples exist in which a clear link between PDI family members and ERAD or an ERAD-like phenomenon has been established. For example, in yeast the chaperone activity of Pdi1 is required for the recognition and degradation of an ERAD substrate, pro- α factor (p α f), which lacks disulfide bonds (Gillece *et al.*, 1999), and Eps1, appears to help target a misfolded membrane protein, Pma1-D378N, for ERAD (Wang and Chang, 2003). In mammalian cells, ERdj5, a protein that contains both a J-domain that interacts with BiP and six thioredoxin repeat motifs, may reduce disulfide bonds to facilitate ERAD substrate retrotranslocation (Ushioda *et al.*, 2008; Hagiwara *et al.*, 2011). Further, some PDIs bind to select bacterial toxins and viruses prior to their retrotranslocation and delivery to the cytoplasm in infected cells (Magnuson *et al.*, 2005; Forster *et al.*, 2006; Schelhaas *et al.*, 2007; Rainey-Barger *et al.*, 2009; Moore *et al.*, 2010; Taylor *et al.*, 2011).

As mentioned above, mammalian PDI is one component of the MTP complex that helps load lipids onto ApoB (Hussain *et al.*, 2003), but it is unknown if PDI targets the protein for ERAD under lipid-poor conditions. Two other mammalian PDI family members, ERp57 and ERp72, can be coprecipitated with ApoB (Adeli *et al.*, 1997; Linnik and Herscovitz, 1998; Hussain *et al.*, 2003; Zhang and Herscovitz, 2003), but their roles during ApoB biogenesis are also unknown. Using both yeast and mammalian cell systems, I now report on the specific action of PDI family members during the ERAD of ApoB. I also show that the degradation of ApoB and CPY*, a soluble ERAD substrate, require different Pdi1-encoded functions in yeast: ApoB utilizes Pdi1's chaperone activity, whereas CPY* utilizes the thioredoxin-like motifs. I

then discovered that Pdi1 is dispensable for the turnover of another ERAD substrate, the alpha subunit of the epithelial sodium channel (α -ENaC), and with the exception of Mpd1 the genes encoding the remaining three PDI relatives can be ablated without an effect on the ERAD of any of the substrates examined in this study. Finally, I found that two human PDI family members also facilitate the ERAD of ApoB in hepatic cells, although PDI—which is a component of the MTP complex—enhanced ApoB secretion. These data demonstrate the diverse PDI activities that are required during ERAD and that different PDI family members can act either to fold or degrade ApoB.

2.1 MATERIALS AND METHODS

2.1.1 Yeast strains, strain construction, and growth assays

Yeast strains were grown at 26°C using standard conditions for growth, media preparation, and transformation unless otherwise noted (Adams *et al.*, 1997). A complete list of yeast strains used in this study can be found in Table 2.

To create the PDI single deletion strains (*mpd1 Δ* , *mpd2 Δ* , *eug1 Δ* , and *eps1 Δ*) PCR-mediated gene disruption was employed using the W303 background (Brachmann *et al.*, 1998). In each case, the KanMX cassette was amplified from the pFA6a-KanMX6 plasmid using primers containing twenty nucleotides of homology to the KanMX cassette (underlined) and forty nucleotides of homology flanking the gene to be disrupted. To amplify KanMX for the

disruption of *MPD1* the following primers were used, (forward) 5'- TCC ACT TAA CAC AAT TAG GAG AGA CAA AAT TTG ACA TAT AAG ATT GTA CTG AGA GTG CAC -3' and (reverse) 5'- TGT GTT TAA TTA GAT AAT CAT TGA ATG AGG AAA CGT ACC ACT GTG CGG TAT TTC ACA CCG -3'. To amplify KanMX for the disruption of *MPD2* the following primers were used, (forward) 5'- GTC TAG TGC AAG TAC GTC GGC AAA GTA AAA CAC AAA GGA GAG ATT GTA CTG AGA GTG CAC -3' and (reverse) 5'- TCG GTA TTC GTA AAG TAA AAG ACA GAG CGA AGC TTA TGT TCT GTG CGG TAT TTC ACA CCG -3'. To amplify KanMX for the disruption of *EUG1* the following primers were used, (forward) 5'- ATA TGG CAA TCT CCC AAC AAG CAC CCG CTC ATA TAA TAC CAG ATT GTA CTG AGA GTG CAC -3' and (reverse) 5'- AGA TGT TAA AAA TGT GCA TTA TAT ATG CTT TAT TTA TTG ACT GTG CGG TAT TTC ACA CCG -3'. To amplify KanMX for the disruption of *EPS1* the following primers were used, (forward) 5'- AAA AAT ACT ATC TAT AAA AAC TAG CTG TAA GGC AGC AGC CAG ATT GTA CTG AGA GTG CAC -3' and (reverse) 5'- AGA TAT CAG CAT TCT TTT ATT TTT ATA ACT ACT TAA GCG TCT GTG CGG TAT TTC ACA CCG -3'. The deletions of *MPD1*, *MPD2*, *EUG1*, and *EPS1* were confirmed by PCR.

Strain SRH01 was created using a plasmid shuffle. Briefly, plasmid pSG01 (see below) was transformed into strain M4492, kindly provided by the Schmitt lab (Saarland University). Transformants were plated on medium containing 5-FAA, which selects for cells that have lost the *TRP1* gene. Therefore, the *MPD1-TRP* plasmid from strain M4492 was lost and replaced with a plasmid in which the expression of Pdi1 was driven from its own promoter. Dithiothreitol (DTT) sensitivity was determined by growing cells overnight at 26°C to logarithmic phase in the appropriate selective medium. The number of cells in each culture was normalized and a

dilution series from each culture (1:10, 1:100, 1:1000) was plated on yeast extract-peptone-dextrose (YPD) solid media (pH 5.5) either containing or lacking a final concentration of 5 mM DTT. The plates were incubated at 30°C for 2 days.

Table 2: Yeast strains used in PDI study

Strain	Genotype	Source
W303	<i>MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1</i>	This lab
<i>mpd1Δ</i>	<i>MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, mpd1::KANMX</i>	This study
<i>mpd2Δ</i>	<i>MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, mpd2::KANMX</i>	This study
<i>eug1Δ</i>	<i>MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, eug1::KANMX</i>	This study
<i>eps1Δ</i>	<i>MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, eps1::KANMX</i>	This study
M4492	<i>MATα pdi1::HIS3 Δeps1 Δeug1 Δmpd1 Δmpd2::G418 ura3 trp1 his3 [pBH1800 (MPD1 CEN TRP1)]</i>	Norgaard et al., 2001
SRH01	<i>MATα pdi1::HIS3 Δeps1 Δeug1 Δmpd1 Δmpd2::G418 ura3 trp1 his3 [pSG01]</i>	This study
<i>pdi1Δ[PDI1^{CGHC}-CGHC]</i>	<i>MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, pdi1::HIS3, [pBH1464]</i>	Luz and Lennarz, 1998
<i>pdi1Δ[PDI1^{SGHS}-CGHC]</i>	<i>MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, pdi1::HIS3, [pBH1852]</i>	Luz and Lennarz, 1998
<i>pdi1Δ[PDI1^{CGHC}-SGHS]</i>	<i>MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, pdi1::HIS3, [pBH1630]</i>	Luz and Lennarz, 1998
<i>pdi1Δ[PDI1²²²⁻³⁰²Δ]</i>	<i>MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, pdi1::HIS3, [pRS-Δ222-302]</i>	Gillece et al., 1999
M4130	<i>MATα, pdi1::HIS3, ade2, can1, ura3, leu2, trp1, his3, [pCT37]</i>	Norgaard et al., 2001
<i>ire1Δ</i>	<i>Matα, lys2, his3, leu2, ura3, ire1::KANMX</i>	This lab
SEY6210	<i>MATα, ura3-52, leu2-3,112, trp1-Δ901, his3-Δ200, lys2-801, suc2-Δ9</i>	Nakatsukasa et al., 2001
<i>htm1Δ</i>	<i>MATα, ura3-52, leu2-3,112, trp1-Δ901, his3-Δ200, lys2-801, suc2-Δ9, htm1::HIS3</i>	Nakatsukasa et al., 2001
W303	<i>MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1</i>	Gauss et al., 2011
KKY415	<i>MATα, pdi1-1, his3-11::HIS3-UPRE LacZ, trp1-1, his3-11,15, ura3-1, can1-100, ade2-1, leu2-3,112</i>	Gauss et al., 2011

2.1.2 Plasmids

The plasmids used in this study are shown in Table 3. To assess the degradation of ApoB29 in yeast, pSLW1-B29 was used (Hrizo *et al.*, 2007). To monitor the degradation of CPY*, we utilized pRS316CPY*-3HA, which was kindly provided by the Weissman lab (University of California, San Francisco)(Bhamidipati *et al.*, 2005).

Plasmids pBH1464, pBH1852, pBH1630, and pCT37 (Holst *et al.*, 1997) were generous gifts from the Schmitt lab (Saarland University). Plasmid pSG01 was constructed by subcloning a PstI-BamHI fragment from plasmid pBH1464 into the same sites in pRS314. To study the degradation of p α F, pSM36 was used (Kim *et al.*, 2005). To monitor the degradation of α -ENaC in the non-essential PDI deletion strains, I used plasmid pRS426GPD ENaC-HA (Buck *et al.*, 2010). To measure the degradation of α -ENaC in strains M4492 and SRH01 (Table 2) a methionine repressible α -ENaC plasmid was constructed by a rotation student, Jessica Coblentz. To this end, an EcoRI-XhoI fragment, corresponding to the coding sequence of α -ENaC and the HA epitope tag from plasmid pRS426GPD α -ENaC-HA, was subcloned into the same sites in pRS426MET25 (Mumberg *et al.*, 1994) and was named pRS426MET25 α -ENaC-HA. To assess the degradation of PrA*-Ab-HA, plasmid pKK223 (Kanehara *et al.*, 2010) was kindly provided by the Ng lab (National University of Singapore, Singapore).

Table 3. Plasmids used in the PDI study

Plasmid Name	Notes	Selectable Marker	Reference
pRS316CPY*-3HA	CPY* expression	<i>URA3</i>	Bhamidipati <i>et al.</i> , 2005
pSLW1-B29	ApoB29 expression	<i>URA3</i>	Hrizo <i>et al.</i> , 2007
pBH1800	CEN <i>MPD1</i>	<i>TRP1</i>	Norgaard <i>et al.</i> , 2001
pBH1464	CEN <i>PDI1</i> _{CGHC-CGHC}	<i>TRP1</i>	Holst <i>et al.</i> , 1997
pBH1852	CEN <i>PDI1</i> _{SGHS-CGHC}	<i>TRP1</i>	Holst <i>et al.</i> , 1997
pBH1630	CEN <i>PDI1</i> _{CGHC-SGHS}	<i>TRP1</i>	Holst <i>et al.</i> , 1997
pRS-Δ222-302	CEN <i>PDI1</i> _{222-302Δ}	<i>TRP1</i>	Gillece <i>et al.</i> , 1999
pCT37	Galactose inducible <i>PDI1</i>	<i>URA3</i>	Norgaard <i>et al.</i> , 2001
pFA6a-KanMX6	KanMX	<i>AMP^R</i>	Longtine <i>et al.</i> , 1998
pSG01	CEN <i>PDI1</i> _{CGHC-CGHC}	<i>LEU2</i>	This Study
pRS426GPD ENaC-HA	ENaC alpha subunit expression	<i>URA3</i>	Buck <i>et al.</i> , 2010
pRS426MET25 ENaC-HA	Methionine repressible ENaC alpha subunit expression	<i>URA3</i>	This Study
pSM36-ppafΔG-HA	pdf expression	<i>URA3</i>	Kim <i>et al.</i> 2005
pKK223	PrA*-Ab expression	<i>LEU2</i>	Kanehara <i>et al.</i> 2010
pJJB20	Vector control corresponding to ApoB29	<i>URA3</i>	Hrizo <i>et al.</i> , 2007
pRS316	Vector control corresponding to CPY*	<i>URA3</i>	Bhamidipati <i>et al.</i> , 2005
pcDNA3.1	Vector control corresponding to PDI, ERp57, and ERp72	Neomycin ^R	Invitrogen, Iowa City, IA
pcDNA3.1-hPDI	Human PDI expression	Neomycin ^R	This Study
pcDNA3.1-hERp57	Human ERp57 expression	Neomycin ^R	This Study
pcDNA3.1-hERp72	Human ERp72 expression	Neomycin ^R	This Study

2.1.3 Assays to measure the degradation of ERAD substrates in yeast

To assess the degradation of ApoB29 in yeast, cells transformed with pSLW1-B29 were grown to logarithmic phase ($OD_{600}=0.4-1.0$) overnight at 26°C in synthetic complete medium lacking uracil but supplemented with glucose to a 2% final concentration. The cells were harvested and resuspended in complete medium supplemented with galactose to a final concentration of 2% and were grown for five hours at 26°C to obtain maximal expression of ApoB29. The cycloheximide chase analysis was performed at 30°C as previously described (Hrizo *et al.*, 2007). Total protein was precipitated as described (Zhang *et al.*, 2001) and immediately resolved by SDS-PAGE before immunoblot analysis. ApoB29 was detected using an anti-hemagglutinin (HA)-horseradish peroxidase conjugated (HRP; clone 3F10; Roche) antibody. Immunoblots were also probed with anti-glucose-6-phosphate dehydrogenase (G6PD) (Sigma-Aldrich, St. Louis, MO) antiserum as a loading control. The G6PD primary antibody was decorated with donkey HRP-conjugated anti-rabbit IgG secondary antibody (GE Healthcare, Waukesha, WI). The Supersignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL) was used for the detection of anti-HA ApoB29 in immunoblots and the Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was used for the detection of anti-G6PD in immunoblots. The signals were quantified using a Kodak 440CF Image Station and the associated Kodak 1D software (Eastman Kodak, Rochester, NY).

To monitor the degradation of CPY*, cells expressing pRS316CPY*-3HA were grown in the appropriate selective media overnight at 26°C to logarithmic phase ($OD_{600}=0.4-1.0$). Cycloheximide chase at 30°C, protein precipitation, and SDS-PAGE were performed as previously described (Tran *et al.*, 2011). Immunoblots were probed with an anti-HA-HRP

conjugated (clone 3F10; Roche) antibody to detect CPY*, and with anti-Sec61 antiserum (Stirling *et al.*, 1992), which served as a loading control. The anti-Sec61 primary antibody was probed with donkey HRP-conjugated anti-rabbit IgG secondary antibody (GE Healthcare, Waukesha, WI). The Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was used to detect anti-HA CPY* and immunoblots were probed with anti-Sec61. The resulting signals were quantified as described above.

The degradation of the α subunit of ENaC (α -ENaC) was assessed by introducing plasmid pRS426GPD α -ENaC-HA (see above and Table 3) into the recipient strains that lacked single, non-essential PDI family members. The cells were grown in selective medium overnight at 26°C to logarithmic phase, and cycloheximide chases, protein precipitation, and SDS-PAGE were performed as described (Buck *et al.*, 2010). α -ENaC degradation in strains M4492 and SRH01 was determined by introducing plasmid pRS426MET25 α -ENaC-HA (see above), which is engineered for the methionine repressible expression of α -ENaC. The cells were grown at 26°C in selective medium with 2mM methionine overnight to logarithmic phase. To obtain maximal expression of α -ENaC, cells were harvested and resuspended to an initial concentration of 0.5 A₆₀₀/mL in selective medium lacking methionine and were grown for another 1.5 h. Cycloheximide chases, protein precipitation, and SDS-PAGE were performed as published (Buck *et al.*, 2010). Immunoblots were probed with an anti-HA-HRP conjugated antibody (clone 3F10; Roche) to detect α -ENaC, and with anti-G6PD antiserum, which served as a loading control. The anti-G6PD primary antibody was probed with donkey HRP-conjugated anti-rabbit IgG secondary antibody (GE Healthcare, Waukesha, WI). The Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was used to detect the anti-HA- α -ENaC and anti-G6PD antibodies on the immunoblots and the signals were quantified as described above.

The ERAD of p α F was measured using plasmid pSM36 (see Table 3 and above). In brief, cells were grown in selective medium at 26°C overnight to logarithmic phase, and were harvested and resuspended to 0.5 A₆₀₀/mL. Next, the cells were pre-incubated at 30°C with vigorous shaking for 10 min and then protein synthesis was stopped by the addition of cycloheximide to a final concentration of 50 μ g/mL. At the indicated time points, 1 mL of cells were harvested and frozen in liquid nitrogen. Total protein was precipitated as described (Zhang *et al.*, 2001) and was resolved by SDS-PAGE followed by Western blot analysis. Immunoblots were probed with an anti-HA-HRP conjugated (clone 3F10; Roche) antibody to detect p α f, and with anti-G6PD antiserum, which served as a loading control. The anti-G6PD primary antibody was probed with donkey HRP-conjugated anti-rabbit IgG secondary antibody. The Supersignal West Pico Chemiluminescent Substrate was used to detect the bound antibodies and the signals were quantified as above.

To monitor the degradation of PrA*-Ab, cells expressing pKK223 were grown in the appropriate selective media overnight at 26°C to logarithmic phase (OD₆₀₀=0.4-1.0). A cycloheximide chase at 30°C, protein precipitation, and SDS-PAGE were performed as described above. Immunoblots were probed with an anti-HA-HRP conjugated antibody to detect PrA*-Ab, and with anti-G6PD antiserum, which served as a loading control. The anti-G6PD primary antibody was probed with donkey HRP-conjugated anti-rabbit IgG secondary antibody (GE Healthcare, Waukesha, WI). The Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was used for detection of anti-HA PrA*-Ab and anti-G6PD immunoblots and the signals were quantified as described above.

2.1.4 Coimmunoprecipitation assay

Disulfide conjugated proteins were immunoprecipitated using a previously published protocol with minor modifications (Sakoh-Nakatogawa *et al.*, 2009). In brief, spheroplasts were prepared from cells expressing ApoB29 or CPY*, as described (McCracken and Brodsky, 1996) and were resuspended in buffer containing 1.2 M sorbitol for 30 min at 30°C. Proteins were precipitated with 10% trichloroacetic acid (TCA), centrifuged at 15,000g for 5 min at 4°C, and resuspended in 2% SDS, 20 mM HEPES-KOH, pH 7.4, 50 mM NaCl, and 35 mM iodoacetamide with a protease inhibitor cocktail. The solution was incubated at 75°C for 5 min and insoluble material was removed by centrifugation at 15,000g for 5 min at 4°C. The supernatant was diluted 10-fold with 20 mM HEPES-KOH, pH 7.4, and 50 mM NaCl, with protease inhibitors, and incubated with anti-HA conjugated resin (Roche) or Sepharose 6B resin (Sigma-Aldrich), as a negative control, for 3 h at room temperature. The immunoprecipitates were washed three times with 20 mM HEPES-KOH, pH 7.4, 50 mM NaCl, and eluted in sample buffer prepared with 120 mM of freshly added DTT for 3 min at 75°C for SDS-PAGE, as described above. Prior to the immunoprecipitation, 1% of the lysate was retained and loaded as a control. The samples were immunoblotted with anti-Pdi1 antibody (a kind gift from V. Denic, Harvard University) and the primary antibody was probed with donkey HRP-conjugated anti-rabbit IgG secondary. The Supersignal West Pico Chemiluminescent Substrate was used for detection. ApoB was detected as described above.

2.1.5 Cysteine modification assay

Cysteines were modified by the addition of maleimide-PEG5000 using a previously published protocol (Tetsch *et al.*, 2011) that we adapted for yeast. Briefly, spheroplasts were prepared from cells expressing ApoB29 and were resuspended in 20 mM HEPES, pH 6.8, 150 mM KOAc, 5 mM MgOAc, 1.2 M sorbitol. Iodoacetamide was added to a final concentration of 10 mM and cells were incubated at 30°C for 15 min. Following incubation, proteins were precipitated by the addition of TCA to a final concentration of 10% and were incubated on ice for 30 min. The cells were centrifuged (16,000g, 4°C, 15 min) and the pellets were resuspended in denaturing buffer (6 M urea, 200 mM Tris-HCl, pH 8.5, 10 mM EDTA, 0.5% SDS) containing 1 mM DTT and were incubated at 37°C for 1 hr. The samples were then divided into two tubes and again proteins were precipitated with 10% TCA on ice for 30 min. Following centrifugation (15 min, 4°C, 16,000g) and removal of residual TCA, the pellets were resuspended in either denaturing buffer (see above) or denaturing buffer with 10mM maleimide-PEG5000 (Layson Bio Inc., Alabama). Samples were incubated at 37°C for 1 h. TCA was added again to a final concentration of 10% and the samples were kept on ice for 15 min before centrifugation (15 min, 4°C, 16,000g). After removal of all TCA, pellets were resuspended in denaturing buffer and 2x non-denaturing sample buffer was added. Samples were heated to 75°C for 5 min and subjected to SDS-PAGE and immunoblotting. Pdi1 and ApoB were detected as described above.

2.1.6 PDI, ERp57, and ERp72 expression and ApoB analysis in rat hepatoma cells

The genes encoding human PDI, ERp57, and ERp72 cDNA were obtained from Open Biosystems Inc. (Huntsville, AL MHS1011-98054157, MHS1011-98054265, MHS1011-75436 respectively). The cDNA was amplified using standard PCR conditions and the following primers, which contained a Kozak consensus sequence (in bold) and KpnI or XbaI restriction sites (underlined). PDI was amplified using the primers (forward) 5'- G TAC GGT ACC **ACC GCC ACC** ATG CTG CGC CGC GCT CTG CTG-3' and (reverse) 5'- G TAC TCT AGA TTA CAG TTC ATC TTT CAC AGC-3'. ERp57 was amplified using the primers (forward) 5'-G TAC GGT ACC **ACC GCC ACC** ATG CGC CTC CGC CGC CTA GCG-3' and (reverse) 5'-G TAC TCT AGA TTA GAG ATC CTC CTG TGC CTT C-3'. ERp72 was amplified using the primers (forward) 5'-G TAC GGT ACC **ACC GCC ACC** ATG AGG CCC CGG AAA GCC TTC CTG-3' and (reverse) 5'-G TAC TCT AGA TTA TCA AAG CTC TTC CTT GGT CCT G-3'. The engineered restriction sites at the 5' and 3' ends allowed for subcloning into the pcDNA3.1 vector. High fidelity amplification and cloning was confirmed by sequence analysis.

Rat hepatoma McArdle (McA-RH7777) cells (ATCC CRL-1601) were cultured at 37°C in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 10% horse serum, L-glutamine, and penicillin/streptomycin. Cells were transfected with 2µg of pcDNA3.1 vector (control), pcDNA3.1-hPDI expression vector, pcDNA3.1-hERp57 expression vector, or pcDNA3.1-hERp72 expression vector, prepared as described above, using FuGENE HD transfection reagent according to the manufacturer's specifications (Roche). A total of 24 h after the initial transfection a second transfection with 2µg of DNA was performed. A pulse-chase analysis was performed 48 h after the second transfection, as previously described (Gusarova *et al.*, 2001). The proteasome-dependence of ApoB100 degradation and secretion was performed

as described (Meex *et al.*, 2011) with either dimethyl sulfoxide (DMSO) or the proteasomal inhibitor MG132 (Z-Leu-Leu-Leu-al; Sigma, St. Louis, MO) added to a final concentration of 25 μ M.

2.2 RESULTS

2.2.1 Pdi1 is the major contributor to ERAD in yeast

Disulfide bond formation is critical for ApoB maturation and secretion (Yang *et al.*, 1989; Yang *et al.*, 1990; Burch and Herscovitz, 2000). In addition, as described in Section 1.3.3, PDI is a component of the MTP complex and two PDI family members, ERp57 and ERp72, have been found associated with ApoB (Adeli *et al.*, 1997; Linnik and Herscovitz, 1998; Hussain *et al.*, 2003; Zhang and Herscovitz, 2003). In order to perform a systematic analysis of the roles of PDIs in the ERAD of ApoB, I first examined ApoB stability in the yeast *Saccharomyces cerevisiae* in which the genes encoding the nonessential PDIs were deleted. Because the wild type yeast strain W303 provided a more consistent read-out for the extent of ApoB degradation than the BY4742 strain (Figure 7), I constructed all of our mutants and performed each of the following experiments in this background (Table 2).

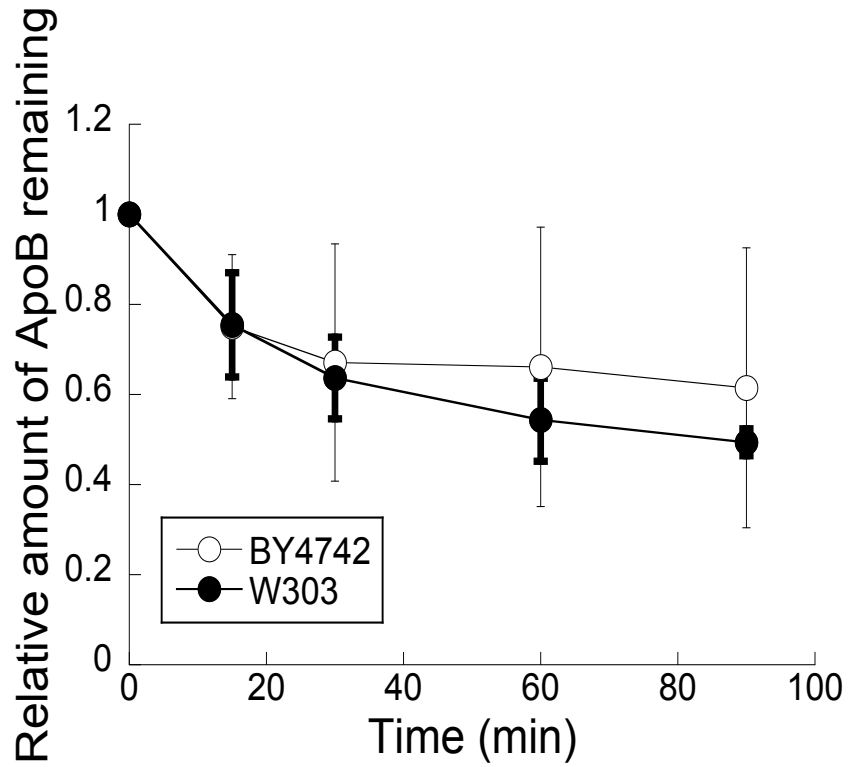


Figure 7: ApoB is degraded more reproducibly in the W303 yeast background than in BY4742

Cycloheximide chase reactions were performed as described in Materials and Methods in two different wild type yeast strains, BY4742 (o) and W303 (•) expressing ApoB29. Chase reactions were performed at 30°C, and lysates were immunoblotted with anti-HA antibody. Anti-G6PD antiserum was used as a loading control. Data represent the means of five experiments, \pm S.D. The bold error bars correspond to W303 and the light error bars correspond to BY4742.

The gene encoding Pdi1 is essential (Farquhar *et al.*, 1991), so to assess whether any of the other PDI family members were important for the degradation of ApoB, *mpd1Δ*, *mpd2Δ*, *eug1Δ*, or *eps1Δ* cells were transformed with the ApoB expression vector and the degradation rate was measured using a cycloheximide chase assay, as described in the section 2.2.3. For these studies, I chose to express ApoB29 from an inducible reporter; ApoB29 is the shortest ApoB isoform whose degradation is metabolically controlled (Wang *et al.*, 1994; Segrest *et al.*, 2001). ApoB29 also contains seven of the eight disulfide bonds found in full length ApoB (Yang *et al.*, 1990; Harazono *et al.*, 2005). In each mutant, I found that ApoB was degraded at wild type levels (Figure 8A). I also measured the ERAD of a well-characterized substrate CPY*, which is a misfolded version of carboxypeptidase Y that has five disulfide bonds (Wolf and Fink, 1975; Finger *et al.*, 1993). In contrast to ApoB, CPY* was stabilized when *MPD1* was deleted, although no stabilization was evident when the other non-essential PDIs were deleted (Figure 8B).

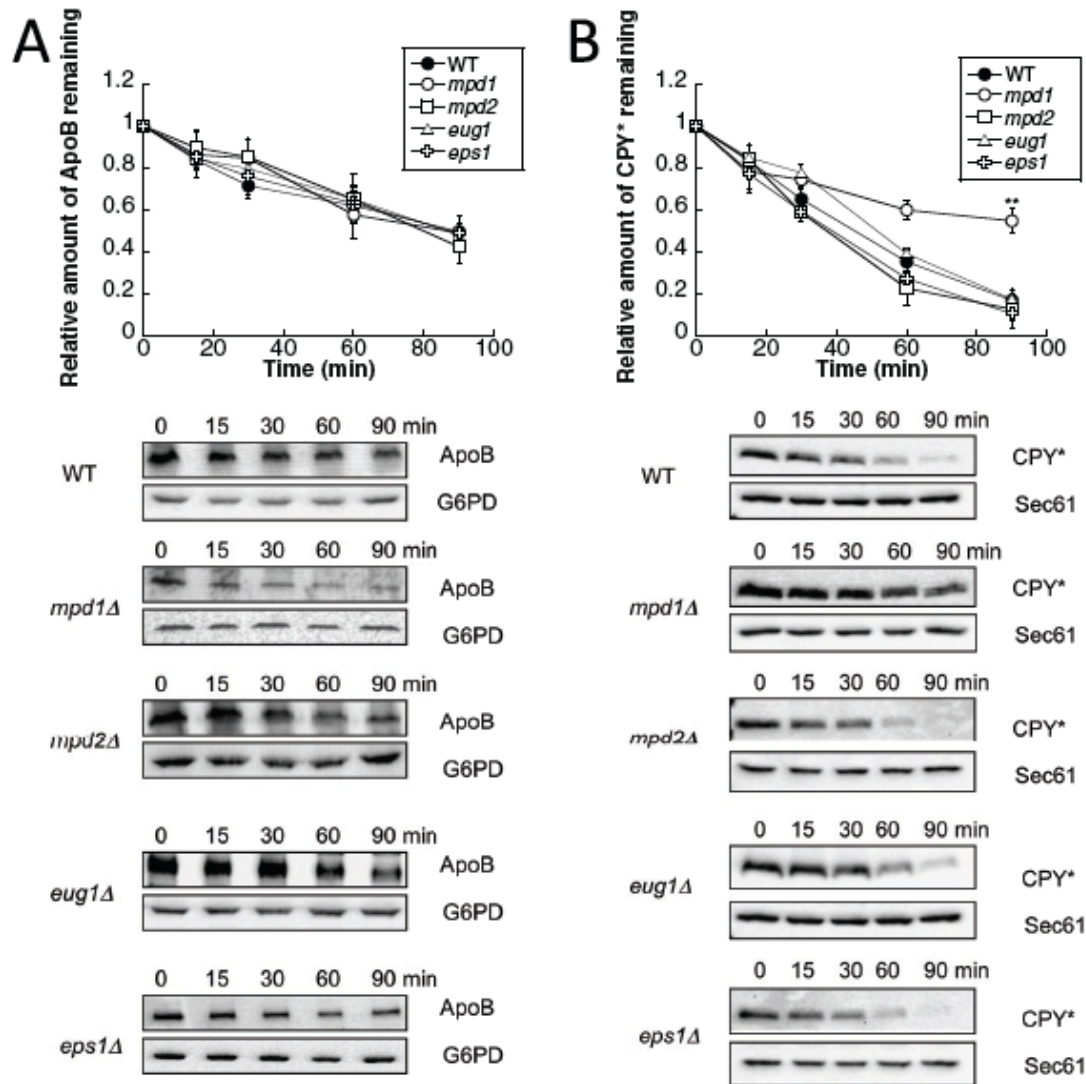


Figure 8: Mpd1 contributes to the ERAD of CPY*, but not ApoB29

Cycloheximide chase reactions were performed as described in the Materials and Methods in wild type (●), *mpd1*Δ (○), *mpd2*Δ (□), *eug1*Δ (△), or *eps1*Δ (⊕) yeast strains expressing ApoB29 (A) or CPY* (B). Chase reactions were performed at 30°C, and lysates were immunoblotted with anti-HA antibody. Anti-Sec61 antiserum was used as a loading control for chase reactions monitoring CPY* turnover and anti-G6PD antiserum was used as a loading control for chase reactions measuring ApoB29 degradation. In the top panels quantitative data are shown, and the bottom panels display representative images. Data represent the means of 4-6 experiments, ± SEM. The lack of visible error bars indicates that the SEM is less than the size of the symbol. Where indicated (**) $p < 0.01$.

Additionally, I examined the degradation of paf, a yeast ERAD substrate that lacks cysteines and that was previously shown to be selected by Pdi1 for degradation (Gillece *et al.*, 1999), as well as α -ENaC, a mammalian protein with seven disulfide bonds whose proteasome- and chaperone-dependent degradation were characterized in yeast (Kashlan *et al.*, 2007; Buck *et al.*, 2010). I found that deletion of the non-essential PDIs also had no effect on the ERAD of these proteins (Figure 9). Combined with previous data demonstrating that Eps1 is required for the ERAD of a mutant form of Pma1, Pma1-D378N, see Section 2.1 (Wang and Chang, 2003), these results indicate substrate specificity amongst the non-essential PDI family members.

I next asked whether yeast Pdi1 facilitates the ERAD of ApoB. Because previous work indicated that Mpd1 over-expression from the *PDII* promoter supported the growth of a *pdi1* Δ mutant (Norgaard *et al.*, 2001), I examined ApoB degradation in *pdi1* Δ *mpd2* Δ *eug1* Δ *eps1* Δ cells in which Mpd1 is the only PDI family member expressed (strain M4492). In this strain, ApoB was completely stabilized (Figure 10A, open circles). I also examined the degradation of ApoB in a strain in which Pdi1 is the only yeast PDI family member expressed (strain SRH01). In these yeast, ApoB degradation was mostly restored (Figure 10A, open squares). I then measured the degradation of CPY* in these strains, since Pdi1 facilitates the ERAD of CPY* (Gillece *et al.*, 1999; Sakoh-Nakatogawa *et al.*, 2009). As with ApoB, CPY* was also significantly stabilized when Mpd1 is the only PDI family member expressed, and protein degradation was again mostly restored when only Pdi1 was expressed (Figure 10B). Because there was a residual degradation defect when ApoB and CPY* turnover were assessed in SRH01, at least one of the other PDI family members may contribute during the process of substrate selection and/or retrotranslocation.

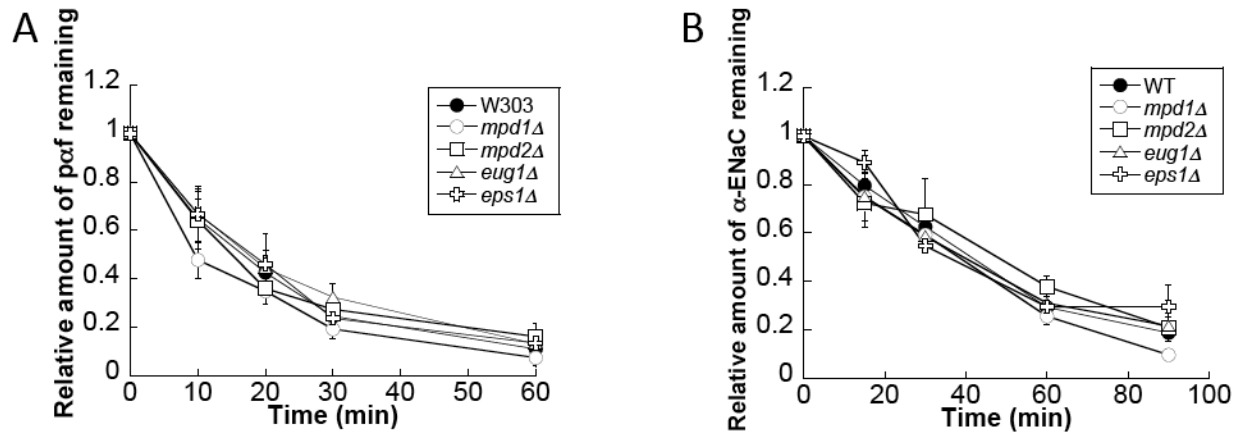


Figure 9: The non-essential PDIs do not contribute to the ERAD of paf or α-ENaC

Cycloheximide chase reactions were performed as described in the Materials and Methods in wild type (●), *mpd1Δ* (○), *mpd2Δ* (□), *eug1Δ* (△), and *eps1Δ* (⊕) yeast strains expressing paf (A) from the pSM36-ppafΔG-HA plasmid or α-ENaC (B) from the pRS426GPD ENaC-HA plasmid. Chase reactions were performed at 30°C, and lysates were immunoblotted with anti-HA antibody. Anti-G6PD antiserum was used as a loading control. Data represent the means of 4-6 experiments, ± SEM. The lack of visible error bars indicates that the SEM is less than the size of the symbol.

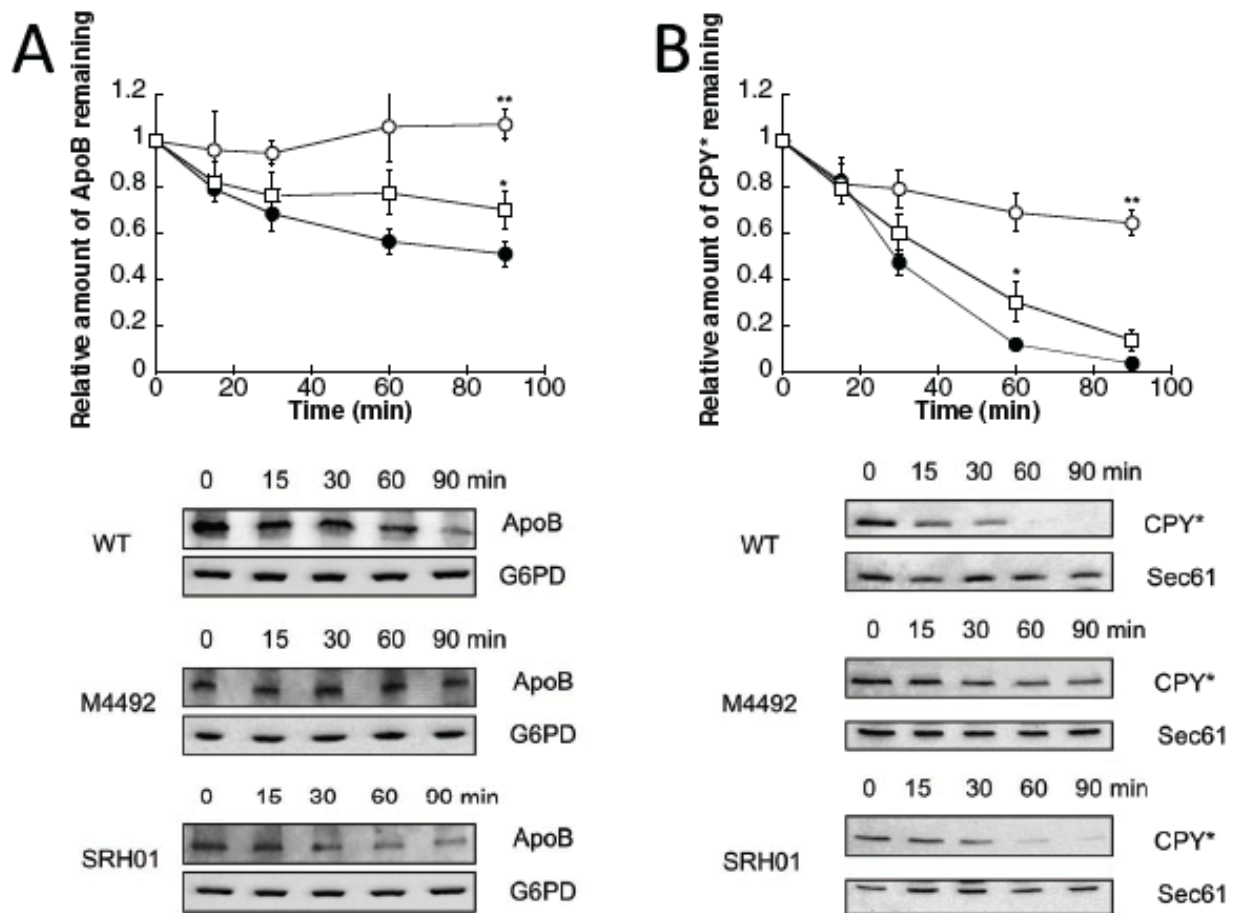


Figure 10: The ERAD of ApoB29 and CPY* is slowed by the loss of *PDI1*

Cycloheximide chase reactions were performed as described in the Materials and Methods in wild type (●), M4492 (*pdi1Δmpd1Δmpd2Δeug1Δeps1Δ* [*MPD1*]) (○), or SRH01 (*pdi1Δmpd1Δmpd2Δeug1Δeps1Δ* [*PDI1*]) (□) yeast strains expressing ApoB29 (A) or CPY* (B). Chase reactions were performed at 30°C, and lysates were immunoblotted with anti-HA antibody. Anti-Sec61 antiserum was used as a loading control for chase reactions monitoring CPY* turnover and anti-G6PD antiserum was used as a loading control for chase reactions measuring ApoB29 degradation. In the top panels quantitative data are shown, and the bottom panels display representative blots. Data represent the means of 4-6 experiments, ± SEM. The lack of visible error bars indicates that the SEM is less than the size of the symbol. Where indicated (*) $p < 0.05$ and (**) $p < 0.01$.

The chaperone activity of Pdi1 was previously found to be important for paf ERAD (Gillece *et al.*, 1999), but the ability of Mpd1 to support the degradation of this substrate as the only expressed PDI family member has not been investigated. I observed measureable stabilization of paf when only Mpd1 was expressed, but degradation was robust when only Pdi1 was expressed (Figure 11A). Interestingly, although α -ENaC has seven disulfide bonds that are important for protein folding and function (Firsov *et al.*, 1999; Kashlan *et al.*, 2011), an ERAD defect was absent when only Mpd1 was expressed (Figure 11B). These data suggest that Pdi1 is not required for the ERAD of all substrates. Normally, ENaC functions as a heterotrimer composed of three subunits, α , β , and γ (Snyder, 2002; Jasti *et al.*, 2007), but in this expression system α -ENaC is an orphan subunit. Therefore, the “decision” to select α -ENaC for degradation may be distinct from other substrates in which intermolecular folding events or protein assembly are monitored. Together, these results suggest that Pdi1 function is not imperative for the ERAD of all substrates, and consistent with previous data (Gillece *et al.*, 1999; Wang and Chang, 2003), my data also indicate that some substrates rely in part on the function of other PDI family members during ER quality control.

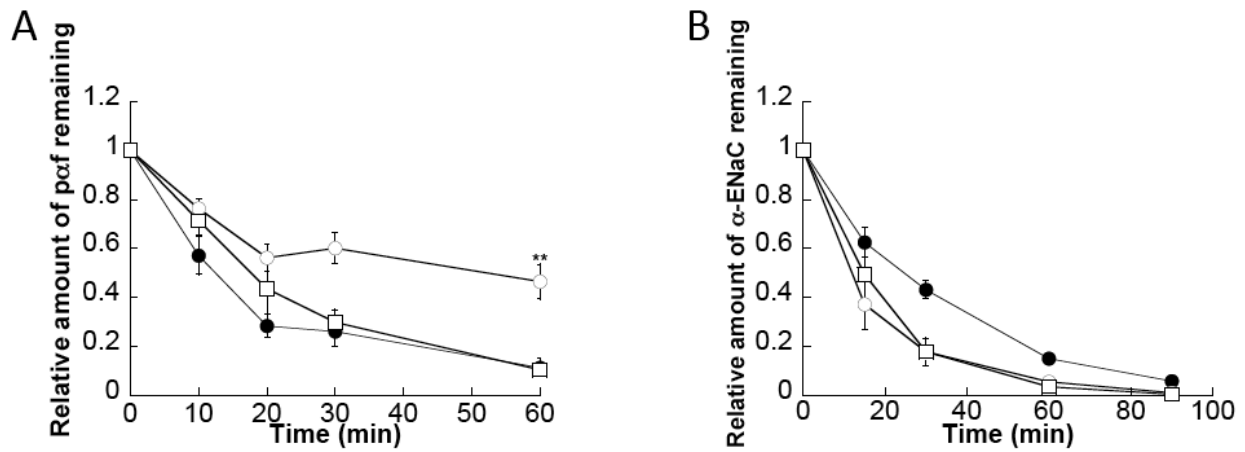


Figure 11: Pdi1 is necessary for the degradation of paf, but not α -ENaC

Cycloheximide chase reactions were performed as described in the Materials and Methods in wild type (●), M4492 (○), or SRH01 (□) yeast strains expressing paf (A) from the pSM36-ppafΔG-HA plasmid or α -ENaC (B) from the pRS426MET25 ENaC-HA plasmid. For expression of α -ENaC, strains were grown overnight at 26°C in selective medium supplemented with 2mM methionine to repress the expression of α -ENaC. The cells were then harvested and resuspended in selective medium without methionine for 90 min to induce the expression of α -ENaC. Chase reactions were subsequently performed at 30°C, and lysates were immunoblotted with anti-HA antibody. Anti-G6PD antiserum was used as a loading control. Data represent the means of 4-6 experiments, \pm SEM. The lack of visible error bars indicates that the SEM is less than the size of the symbol. Where indicated (**) $p < 0.01$.

2.2.2 ApoB and CPY* associate with Pdi1, and their degradation requires either the chaperone activity or the thioredoxin-like motifs

I next assessed if Pdi1 directly associates with ApoB and CPY*. To this end, the ability of Pdi1 to form a mixed disulfide with the ER luminal protein, Htm1/Mnl1, was assessed (Sakoh-Nakatogawa *et al.*, 2009). In this protocol, yeast cells expressing ApoB are spheroplasted and lysed, and then proteins are precipitated under conditions in which preformed disulfide bonds are trapped (see Section 2.2.4). I therefore precipitated ApoB under these conditions and asked whether Pdi1 bound to this substrate after adding reductant and performing SDS-PAGE. When ApoB was immunoprecipitated, Pdi1 was also present, but an abundant, cytosolic protein (G6PD) that contains a single cysteine was absent (Figure 12A, lane 5). I will note that a minor amount of Pdi1 associated with resin when strains lacked ApoB (lane 6), but neither ApoB nor PDI were precipitated using unconjugated resin, indicating a specific association between Pdi1 and ApoB. Consistent with Pdi1 having a direct effect on the degradation of CPY*, I also observed a Pdi1-CPY* interaction (Figure 12B, lane 5). A prominent association between these proteins was absent in precipitations from lysates that lacked the substrate (Figure 12B, lane 6) or in mock precipitations (Figure 12B, lanes 3 and 4), although here too a small amount of Pdi1 appears to nonspecifically associate with the resin. Nevertheless, these results strongly suggest that ApoB and CPY* form mixed disulfides with Pdi1.

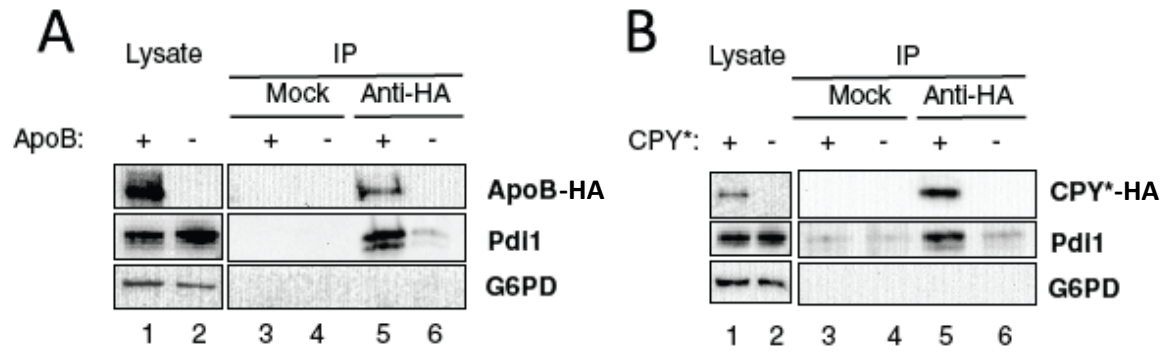


Figure 12: Pdi1 physically interacts with ApoB29 and CPY*

Native immunoprecipitation reactions were performed using anti-HA resin, or unconjugated Sepharose (“Mock”), using lysates from wild type yeast strains expressing ApoB29 (A) or CPY* (B). In both panels, cell lysates were also examined that contained a vector control (“-”) in place of the ApoB29 (“+”) expression vector. A total of 1% of the input for the precipitation was also examined (“Lysate”). After precipitation and SDS-PAGE, the indicated proteins were examined by immunoblot analysis.

Because Pdi1 interacts with ApoB and facilitates its degradation, I next investigated which Pdi1-embedded function was important for ApoB ERAD. I first asked whether the a or a' thioredoxin-like active site was necessary. To this end, strains were used in which *PDII* had been deleted, but the cells expressed wild type *PDII* on a plasmid (*PDII*_{CGHC-CGHC}), *PDII* on a plasmid with both cysteines in the a active site mutated to serines (*PDII*_{SGHS-CGHC}), or *PDII* on a plasmid with both cysteines in the a' active site mutated to serines (*PDII*_{CGHC-SGHS}). ApoB degradation was then measured by cycloheximide chase analysis. When either active site's cysteines were mutated, ApoB was degraded at wild type levels (Figure 13A). In contrast, when either active site was mutated, CPY* was completely stable (Figure 13B), indicating that both thioredoxin-like motifs—and their redox activities—are necessary for CPY* but not ApoB degradation.

Another interpretation of the data presented in Figure 13A is that the a and a' sites function redundantly to support ApoB degradation; however, it is thought that the two sites are not equivalent (Holst *et al.*, 1997; Hatahet and Ruddock, 2009; Wang *et al.*, 2009; Vitu *et al.*, 2010), and at least one of the thioredoxin motifs must be capable of forming a thiolate to support cell viability (Laboissiere *et al.*, 1995; Chivers *et al.*, 1996). A more likely possibility is that Pdi1's chaperone activity, which mediates Pdi1's ability to bind some peptides in the ER (Klappa *et al.*, 1998; Gillece *et al.*, 1999), is necessary for ApoB degradation. To test this hypothesis, I used strains in which *PDII* was deleted, but the cells expressed either wild type *PDII* on a plasmid or *PDII* with its b' chaperone domain deleted (*PDII*_{222-302Δ}). As shown in Figure 13C, ApoB was completely stable when Pdi1's chaperone domain was absent.

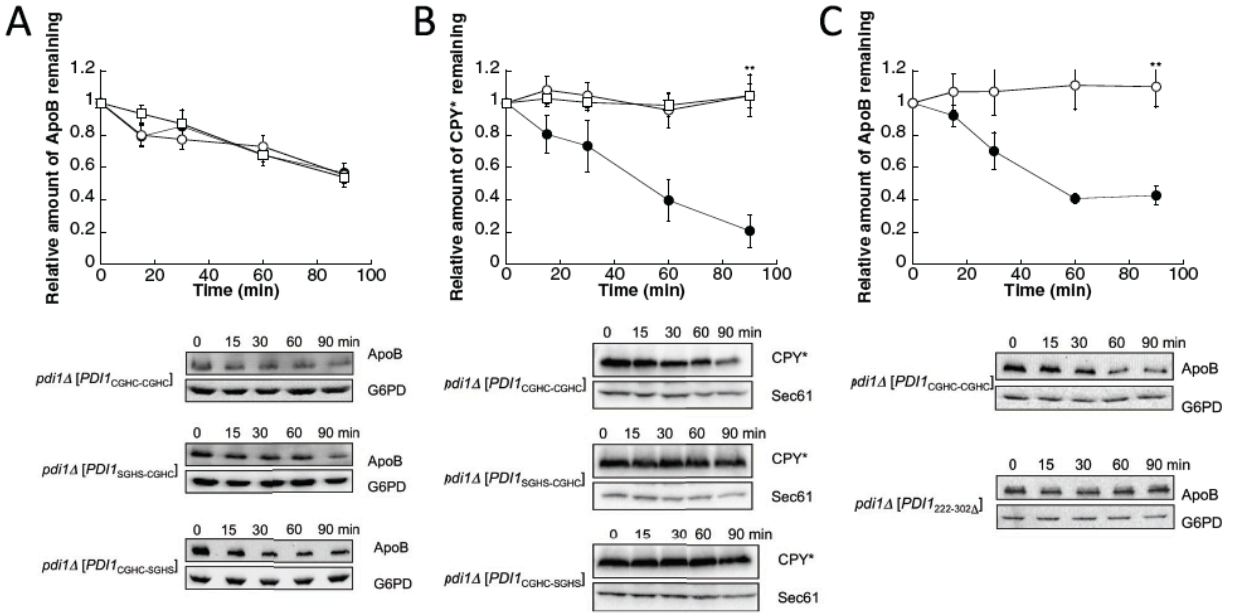


Figure 13: The chaperone-like activity and the oxidoreductase activity of Pdi1 respectively facilitate the ERAD of ApoB29 and CPY

Cycloheximide chase reactions were performed as described in the Materials and Methods in *pdi1Δ* [*PDII*_{CGHC-CGHC}] (●), *pdi1Δ* [*PDII*_{SGHC-CGHC}] (○), or *pdi1Δ* [*PDII*_{CGHC-SGHC}] (□) yeast strains expressing ApoB29 (A) or CPY* (B). Cycloheximide chase reactions were also performed in *pdi1Δ* [*PDII*_{CGHC-CGHC}] (●) or *pdi1Δ* [*PDII*_{222-302Δ}] (○) yeast strains expressing ApoB29 (C). Chase reactions were conducted at 30°C, and lysates were immunoblotted with anti-HA antibody. Anti-Sec61 antiserum was used as a loading control for chase reactions monitoring CPY* and anti-G6PD antiserum was used as a loading control for chase reactions measuring ApoB29 degradation. In the top panels quantitative data are shown, and the bottom panels display representative blots. Data represent the means of 5-7 experiments, ± SEM. The lack of visible error bars indicates that the SEM is less than the size of the symbol. Where indicated (**) $p < 0.01$.

In order to determine if the chaperone mutant strain supported the ERAD of other substrates, CPY* degradation was measured. Consistent with previous results (Gillece *et al.*, 1999), no CPY* degradation defect was observed when the Pdi1 chaperone domain was mutated (Figure 14A). I also confirmed that the mutant Pdi1 protein was expressed to the same level as wild type Pdi1 and remained stable over the 90 min chase period (Figure 14B). Together, these results indicate distinct requirements for Pdi1-embedded activities during ERAD: some substrates (α -ENaC) are Pdi1-independent, some (ApoB and p α F) require the protein's chaperone activity, and others (CPY*) require both of the enzyme's thoredoxin-like motifs.

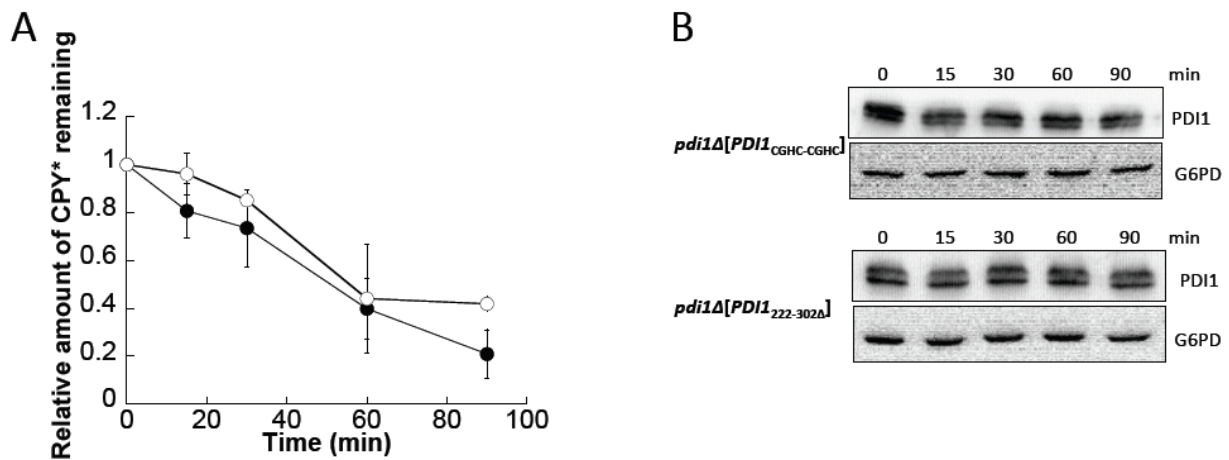


Figure 14: The chaperone activity of Pdi1 is not necessary for the ERAD of CPY*

Cycloheximide chase reactions were performed as described in the Materials and Methods in *pdi1Δ* [*PDI1*_{CGHC-CGHC}] (●) and *pdi1Δ* [*PDI1*_{222-302Δ}] (○) yeast strains expressing CPY* (A). Chase reactions were performed at 30°C, and lysates were immunoblotted with anti-HA antibody. Anti-G6PD antiserum was used as a loading control. Data represent the means of 4-6 experiments, \pm SEM. The lack of visible error bars indicates that the SEM is less than the size of the symbol. Lysates were also immunoblotted with anti-Pdi1 antiserum and representative images are shown (B).

One surprising aspect of this study is that ApoB forms a disulfide crosslinked species with Pdi1 in the yeast ER (Figure 12A). Thus, one might predict that the redox or isomerization activity—but not the chaperone activity—of Pdi1 might have been most important for ERAD. Unexpectedly, mutating the cysteine residues in the a or a' sites had no effect on the rate of ApoB degradation, although CPY* degradation was blocked. There are two explanations for these data. First, there may be two pools of ApoB: One that has formed crosslinks and one that is *en route* for degradation, an event that may require a non-covalent, chaperone-like association between ApoB and Pdi1. In fact, the ERAD of ApoB is incomplete when chase reactions are conducted in yeast (see for example Figure 7), so perhaps the remaining, stable material represents the crosslinked pool. Second, Pdi1's two activities, as a redox enzyme and as a chaperone (LaMantia and Lennarz, 1993; Gillece *et al.*, 1999), may provide unique functions during ApoB biogenesis. For example, Pdi1 may initially form disulfide bonds with ApoB, which may represent an attempt by Pdi1 and other chaperones to assist protein folding. But, because yeast lack the MTP complex, the chaperone activity of Pdi1 then becomes essential to maintain ApoB's retrotranslocation-competence prior to degradation. Consistent with this view, I found that ApoB can crosslink to Pdi1 even if the chaperone domain or the a or a' active sites have been mutated (Figure 15A). I also found that CPY* crosslinks to each Pdi1 species (Figure 15B), which is anticipated since each active site is necessary for CPY* ERAD and because degradation is unaltered in the chaperone mutant, suggesting that Pdi1 and CPY* are still able to interact through the active sites when the b' domain is deleted.

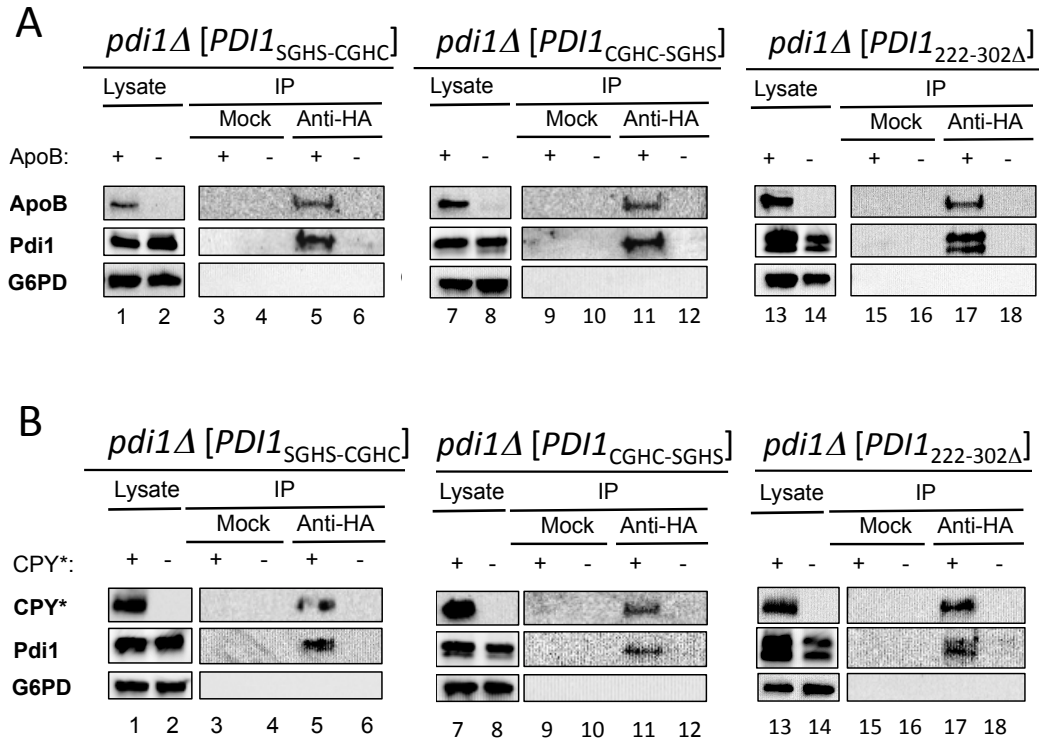


Figure 15. Mutant forms of Pdi1 co-precipitate with ApoB29 and CPY*

Native immunoprecipitation reactions were performed using anti-HA resin, or unconjugated Sepharose (“Mock”), using lysates from *pdi1Δ* yeast strains expressing active site mutant (lanes 1-6 and 7-12) or chaperone mutant (lanes 13-18) forms of *PDI1* on a plasmid. As indicated, each strain also expressed (A) ApoB29 (“ApoB”), (B) CPY* (“CPY*”), or harbored an empty vector control (“-”). A total of 1% of the input for the precipitation was also examined (“Lysate”). After precipitation and SDS-PAGE, the indicated proteins were examined by immunoblot analysis. The doublet observed for Pdi1 in some panels is due to differential glycosylation.

2.2.3 Yeast expressing only Mpd1 or expressing specific mutant forms of Pdi1 are sensitive to DTT

I hypothesized that if the chaperone mutant strain (*pdi1Δ*[*PDII*_{222-302Δ}]) was more sensitive to ER stress and less viable, then the stabilization of ApoB observed in these *Pdi1*_{222-302Δ}-expressing yeast could have resulted from a non-specific effect on induction of ER stress and compromised cell growth. Therefore, the sensitivities of all of the examined mutants to an ER stress-inducing agent, dithiothreitol (DTT), were measured. I first found that the strains with a single deletion of any of the individual non-essential PDIs were DTT-insensitive. In contrast, and as a control, *ire1Δ* yeast, which are unable to induce the unfolded protein response (Cox and Walter, 1996), were inviable when plated on DTT (Figure 16). Strain M4492 (*pdi1Δmpd1Δmpd2Δeug1Δeps1Δ* [*MPDI*]) was significantly sicker than wild type cells on rich media, and was extremely DTT sensitive; however, when *Pdi1* was the only PDI family member expressed (strain SRH01, *pdi1Δmpd1Δmpd2Δeug1Δeps1Δ* [*PDII*]), DTT sensitivity was absent (Figure 16). In accordance with previous data (Holst *et al.*, 1997), I also found that mutation of the a active site in *Pdi1* (*pdi1Δ*[*PDII*_{SGHS-CGHC}]) led to modest DTT sensitivity. Notably, the b' chaperone mutant strain (*pdi1Δ*[*PDII*_{222-302Δ}]) was DTT insensitive. These results suggest that ApoB stabilization in the *Pdi1*_{222-302Δ} chaperone-defective strain does not result from unmitigated stress and poor cell growth.

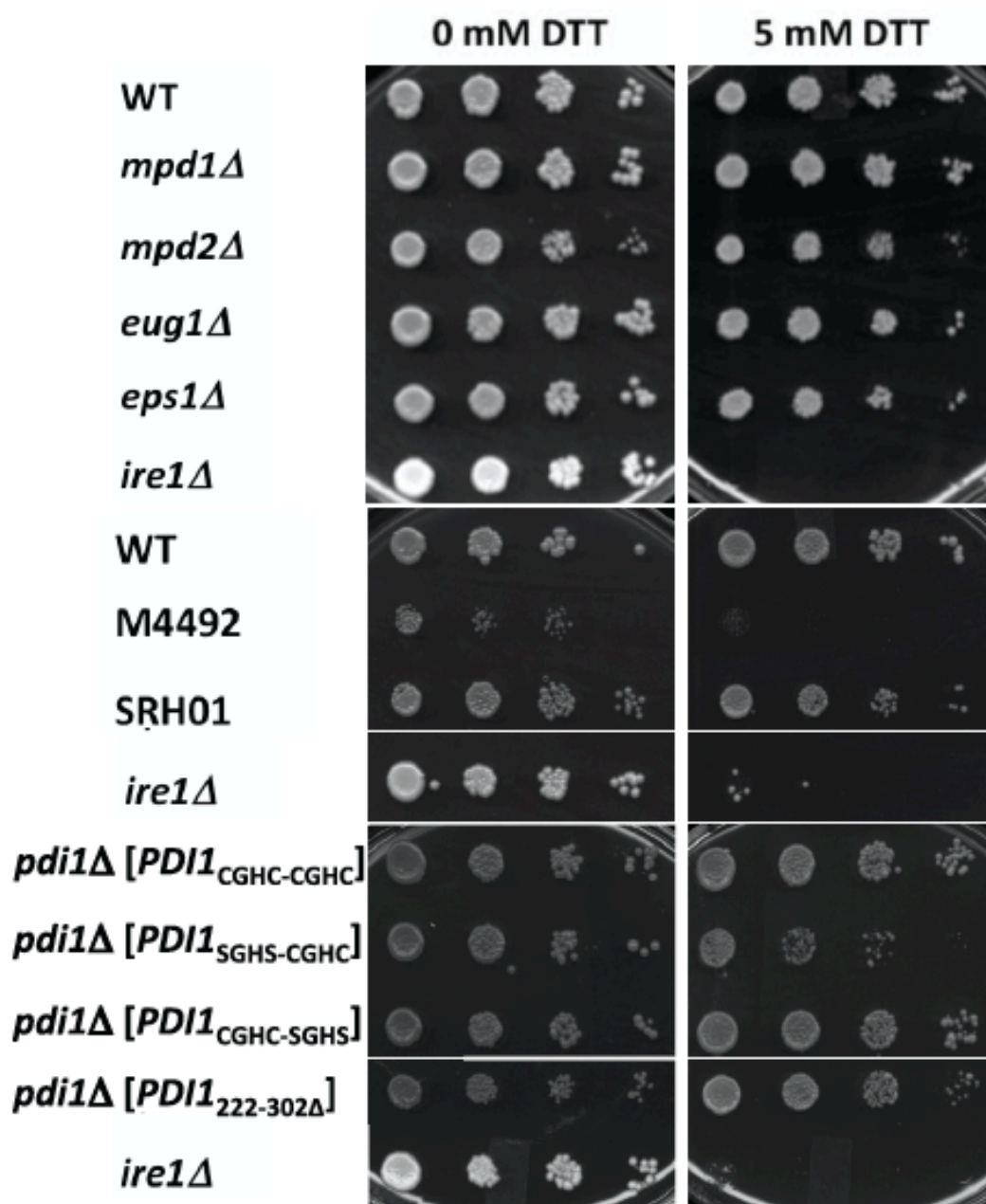


Figure 16: Strains with deletions of or mutations in the PDI family members exhibit varying sensitivities to the reducing agent, dithiothreitol (DTT)

The indicated yeast strains were grown in selective medium to mid log phase. Serial dilutions of the cells were spot plated onto medium containing either 0mM DTT or 5mM DTT, as indicated, and grown for 2 d at 30°C. The data are representative of several independent trials, and in liquid culture the doubling times for the single deletions in the absence of DTT were essentially identical.

2.2.4 ApoB degradation is EDEM independent

Pdi1 interacts with and is required for the oxidation of an intermolecular disulfide bond in Htm1/Mnl1 (Clerc *et al.*, 2009; Sakoh-Nakatogawa *et al.*, 2009), which is the ER degradation enhancing α -mannosidase-like lectin (EDEM) homolog in yeast. As in mammals, Htm1 recognizes misfolded glycoproteins and targets them for degradation (Jakob *et al.*, 2001; Nakatsukasa *et al.*, 2001). Recently, a mutation in *PDII*, *pdi1-1*, was identified in a screen for yeast that require a functioning unfolded protein response for viability (Gauss *et al.*, 2011). The *pdi1-1* mutation harbors a leucine in place of a proline in the center of the b' domain (amino acid 313) and disrupts the interaction of Pdi1 with Htm1. This, in turn, affects the ERAD of select substrates. Formally, then, the delayed degradation of ApoB, a glycoprotein, in the *PDII*₂₂₂₋₃₀₂ Δ strain (Figure 13C) might have arisen from an indirect effect on Htm1 activity.

To address this possibility, I asked whether ApoB degradation was altered in the *pdi1-1* mutant. ApoB was degraded at wild type levels in the *pdi1-1* strain (Figure 17A). As a control for this experiment, PrA*-Ab, a mutated version of the vacuolar protein Proteinase A that requires Htm1 (Finger *et al.*, 1993; Spear and Ng, 2005; Kanehara *et al.*, 2010), was stabilized in *pdi1-1* yeast (Figure 17B). These data suggest that ApoB degradation is Htm1-independent, and that the effect of the *PDII*₂₂₂₋₃₀₂ Δ mutant on ApoB (Figure 13C) was not due to a secondary effect via disrupted association between Htm1 and Pdi1. Consistent with this hypothesis, I also failed to observe an effect on ApoB stability in *htm1* Δ strains compared to wild type yeast (Figure 17C). Further, as shown above, ApoB was degraded at wild type levels in *pdi1* Δ [*PDII* SGHS-CGHC] and *pdi1* Δ [*PDII* CGHC-SGHS] strains (Figure 13A). These mutants have previously been

reported to ablate the function of Htm1 during ERAD (Sakoh-Nakatogawa *et al.*, 2009). Overall, these data indicate that the yeast EDEM homolog is dispensable for ApoB turnover and confirm that ApoB degradation requires Pdi1's chaperone activity.

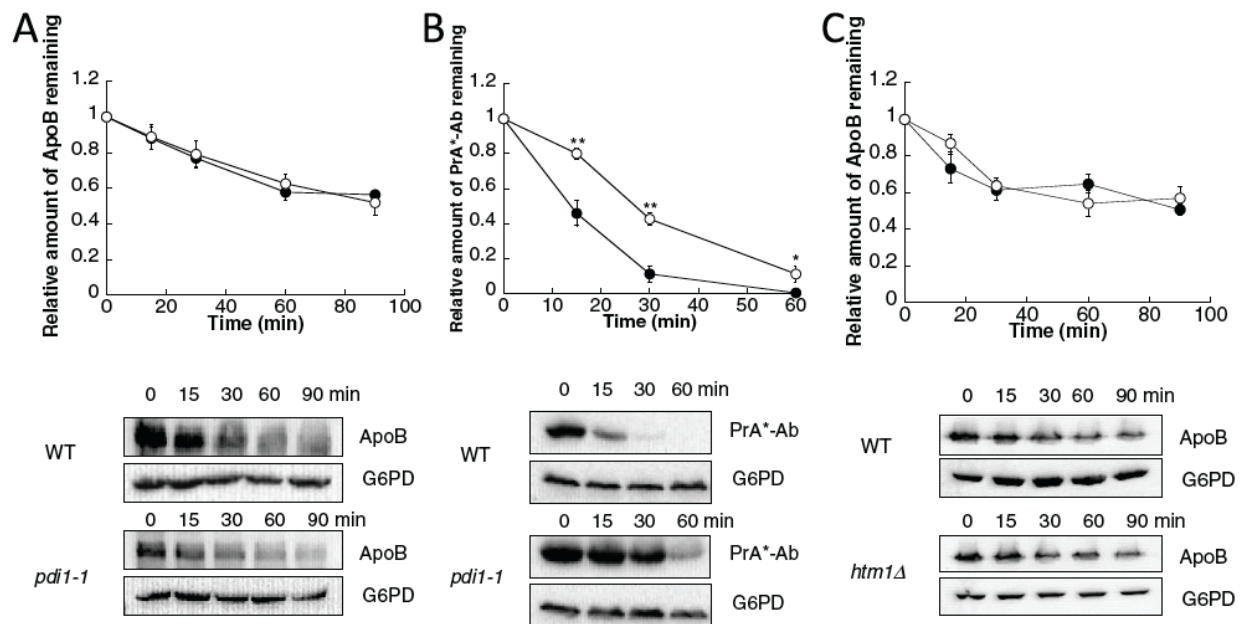


Figure 17: The EDEM homolog Htm1 does not play a role in the degradation of ApoB

Cycloheximide chase reactions were performed as described in the Materials and Methods in wild type W303a (●) or KKY415 (*pdi1-1*) (○) yeast strains expressing ApoB29 (A) or PrA*-Ab (B). Cycloheximide chase reactions were also performed in wild type SEY6210 (●) or *htm1Δ* (○) yeast strains expressing ApoB29 (C). Chase reactions were performed at 30°C, and lysates were immunoblotted with anti-HA antibody. Anti-G6PD antiserum was used as a loading control. Data represent the means of 4-6 experiments, \pm SEM. The lack of visible error bars indicates that the SEM is less than the size of the symbol. Where indicated (*) $p < 0.05$ and (**) $p < 0.01$.

Defects in chaperone function can affect the biochemical properties of an ERAD substrate in yeast (Nishikawa *et al.*, 2001; Kabani *et al.*, 2003). Because the chaperone activity of Pdi1 was required for maximal ApoB turnover, I was curious whether ApoB's conformation was altered in the b' mutant strain. As a read-out for ApoB's conformation in the yeast ER, I chose to measure the reactivity of any free cysteines in ApoB to the thiol-modifying reagent, maleimide-PEG5000, after ApoB isolation and reduction. In previous work, maleimide-PEG5000 was used to quantify the free cysteines that were available in Pdi1 mutants that lacked active site cysteines, and to assess whether the loss of Htm1/Mnl1 association altered cysteine modification (Sakoh-Nakatogawa *et al.*, 2009).

When ApoB was expressed in the *pdi1Δ*[*PDII*_{CGHC-CGHC}], *pdi1Δ*[*PDII*_{SGHC-CGHC}], or *pdi1Δ*[*PDII*_{CGHC-SGHC}] strains and—after reduction—the maleimide-PEG5000-modified protein was detected by immunoblotting, a prominent species at the predicted molecular weight of ApoB29 (~150,000) as well as a high molecular weight “smear” was observed (Figure 18, lanes 2, 4, and 6, compared to samples without maleimide-PEG5000, lanes 1, 3, and 5). These data suggest that there is no gross difference in the degree of modification when the degradation of ApoB is robust in the *pdi1* mutants. Interestingly, when ApoB was expressed in the *pdi1Δ*[*PDII*_{222-302Δ}] strain and was modified with maleimide-PEG5000 after reduction, the magnitude of the high molecular weight pool was significantly reduced (Figure 18, lane 8). This can be seen better in the two separate examples with 5-fold more protein loaded on the gel (Figure 18, lanes 10 and 12). In the *pdi1Δ*[*PDII*_{222-302Δ}] strain, the amount of a lower molecular weight band (~110,000) specific to ApoB also increased (Figure 18, lanes 7). These combined results suggest that when the chaperone domain of Pdi1 is absent, the conformation of ApoB

may be altered such that several of its disulfide bonds are no longer available for maleimide-PEG5000 modification and that the protein becomes more protease susceptible.

A

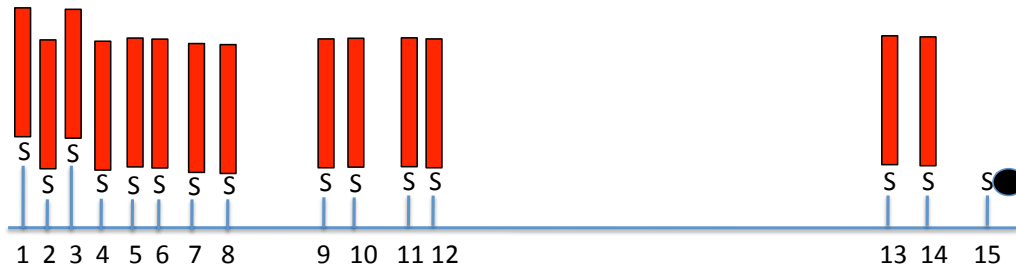
Step #1: Modify free sulfhydryls with iodoacetamide (•)



Step #2: Reduce disulfide bonds into free sulfhydryls using DTT



Step #3: Modify free sulfhydryls using maleimide-PEG5000 (■)



B

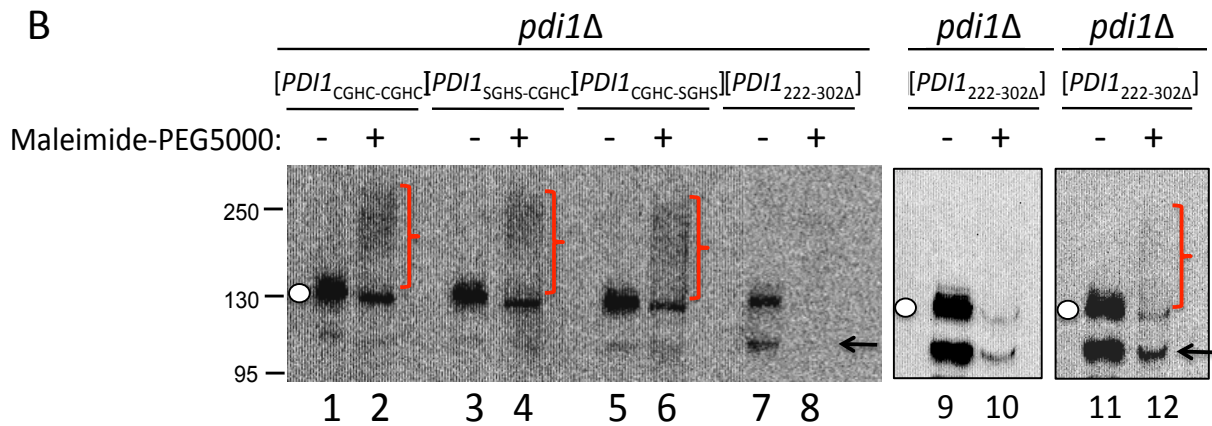


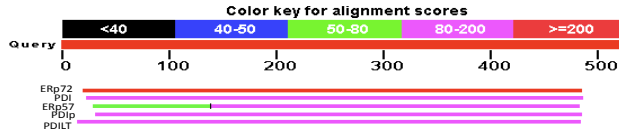
Figure 18. Deletion of the chaperone domain of Pdi1 alters the modification of ApoB by maleimide-PEG5000

(A) A schematic representation of the cysteine modification assay is depicted. The blue line represents ApoB29 with its 15 cysteines. (B) Cysteine modification assays were performed as described in the Materials and Methods. Spheroplasts expressing ApoB29 from *pdi1Δ* cells expressing wild type Pdi1 (*PDI1*_{CGHC-CGHC}) or the indicated mutants first were treated with iodoacetamide followed by DTT, and were then treated with maleimide-PEG5000 (Lanes 2, 4, 6, 8, 10, and 12) or a vehicle control (Lanes 1, 3, 5, 7, 9, and 11). The samples were analyzed by SDS-PAGE and immunoblotted with anti-HA antibodies. Molecular mass markers ($\times 10^{-3}$ Da) are indicated to the left of the gel. The predicted size of ApoB29 is ~150kDa, indicated with (●). Note that lanes 9-12 are from two independent experiments with 5-fold more sample loaded on the gel in order to visualize the smear of modified protein upon the addition of Maleimide-PEG5000 in the chaperone mutant strain (lanes 10 and 12). Maleimide-PEG5000 modified protein is indicated with a red bracket. The protease susceptible degradation product is indicated with a black arrow.

2.2.5 Distinct PDI family members contribute differently to ApoB biogenesis in hepatic cells

To assess whether the knowledge acquired from the use of the yeast system might indicate which mammalian chaperones similarly mediate ApoB quality control, I examined the contributions of select mammalian PDI family members on ApoB biogenesis in hepatic cells. While there are only five yeast PDI family members, mammals express twenty PDI-like proteins (Hatahet and Ruddock, 2009). In order to identify which of these PDIs to examine, I first performed a BLAST search with yeast Pdi1 against the human protein database. The three best hits were ERp72, PDI, and ERp57 (Figure 19).

A



B

Erp72

Range 1: 168 to 635			36nPerit	Graphics	▼ Next Match	Previous Match
Score	Expect	Method	Identities	Positives	Gaps	
205 bits(522)	5e-57	Compositional matrix adjust.	158/499(32%)	243/499(48%)	67/499(13%)	
Query 22	AQGEAVAPEDSAVVKLATDSFNEIYQSHDLVLAFFAPWCGHCKNMAPEYVKAETLVEK					
Sbjct 168	+Q + P + +V L ++F+E + D+L EF+APWCGHCK +APEY KAA+ L ++					81
Query 82	N--ITLAQDCTENQDLCMEHNI PGFPSLKIFKNSDVNNSIDYEGPRTAEIVQFMIKQS					139
Sbjct 227	+ I LA+D T DL ++ G+P+LKIF+ DY GPR IV +MI+QS					283
Query 140	QPA--VAVVADLPAYLANETFTVTPVIVQSGKIDAFNATFYSMANKHNDYDFVSAENA					196
Sbjct 284	GPSSKELLTKQVLEKQKGDGVIIIGVFKESIDAPQ-QYQADANQDFVHFHFTST					342
Query 197	DDDFKLSIYLPASAMDEPVVYNGKKADIADADVFEKWLQVEALPYFGIDGSVFAQVYES-					255
Sbjct 343	E----IAKFLKVSQGLVVMQFEK-----FQS--KYEPKSHMDVQSGTQDSAIKDF					303
Query 256	-----GLFL-CYGLFVSEDEEELEYKPLFELAKKNRGLMNVSID-----AKGFKG					388
Sbjct 389	VLKYLFLVGHKRVSNDAKRYTRFL-----VVVYSVDFSDYRAATQFWR-SK					437
Query 304	NLNKQEFF--LFAIHD-----MTDELKYLGLPQLSEEA----FDELSKIVLE----SKA					348
Sbjct 438	L + PP FAI D E GL + E+ DE K +E S					497
Query 349	IESLVKDFLKGDSAPVKSQEIFENQDSVFLQVGNHDEIVNDPKDVLVLYAPWCGH					408
Sbjct 498	+ V F KG P++KQ+ +N V -VGR D IV DPKRDV+ +YAPWCGH					557
Query 409	CKRLAPYQELADTYANATSDVLI AKLDHTENDVRG--VVEGYPTIVLYPGGKSSSEV					466
Sbjct 558	CKQLPEVYNSIAKYY-KGQGLVIAKMDATANDVPSDRYVEGFTTYIFAPSGDKNPFK					616
Query 467	YQGS-RSLDSLFDFIKENG 484					
Sbjct 617	FEQDRLDHLKSKFIEEHA 635					

C

PDI

Score	Expect	Method	Identities	Positives	Gaps
196 bits(497)	2e-54	Compositional matrix adjust.	140/479(29%)	235/479(49%)	49/479(10%)
Query 32	SAVVKLATDSFNEIYQ---SHDLVLAFFAPWCGHCKNMAPEYVKAETLVEKNITLAIQI				88
Sbjct 25	S V++L D+F I S L+L EFFAPWCGHCK +APEY +AA T ++ + LA++				83
Query 89	DCTENQDLCMEHNI PGFPSLKIFKNSDVNNSIDYEGPRTAEIVQFMIKQSPAVAVVAD				148
Sbjct 84	DCTANTWTCNKYGVSGYPTLKIIFRDGEESGA--YDGPRTADGIVSHLAKQAFASVPLKS				141
Query 149	LPAYLANETFTV----PVIQSGKIDAFNATFYSMANKHNDYDFVSA-----ENAD				197
Sbjct 142	EEF+---EFTISKDASVVGFFKDLFEASHSEFLKAASNLRDYFAHTNVESLVNKYDD				198
Query 198	DDFKLSIYLPASAM----DEPVVYNGKKADIADADVFEKWLQVEALPYFGIDGSVFAQY				252
Sbjct 199	D GEGITLFRPSHLNFKEDKTVAYTEQKMTSGIKRFIQENIFGICPHMTEDNKDLL---				255
Query 253	VESGLGLYLFYNDDEELEYKPLFTLAKKNRGLMNFVSDARKF---GRH-----AGN				304
Sbjct 256	---GQKDLLIAYDYD-----YEKNAGSNYWRNRVNMVAKFLDAGQKLHFAVASR				304
Query 305	LNKMQFFLFAIHDHMTDELKYLGLPQLSEAFDELSKIVLESKAIETLVKDFLKGDSAPI				364
Sbjct 305	KTFSHLSDFGLSESTGEIPVAVRTAKGEKFMQEEFSDGKALERFLEDYDGNLKRY				364
Query 365	VKSQEIFENQDSVFLQVGNHDEIVNDPKDVLVLYAPWCGHCKRLAPTYQELADTYA				424
Sbjct 365	+KS+ I E+ D V +V +N DEIVN+ KQVL+ +YAPWCGHCK L P Y+EL +				423
Query 425	NATSDVLI AKLDHTENDVRGVV-IEGYPTIVLYPGGKSSSEVYQGSRLDSLFDFIKE				482
Sbjct 424	RKDPNVIAKMDATANDVPSYVEGFTTYIFSPANKNQKPKYEGRELSDFISYLKR				482

D

Erp57

Range 1: 16 to 475 GenPept Graphics			Next Match & Previous Match		
Score	Expect	Method	Identities	Positives	Gaps
194 bits(493)	7e-54	Compositional matrix adjust.	151/488(31%)	241/488(49%)	55/488(11%)
Query 25	EAVAPEDS-AVVKLATDSFNEIYQSHDLVLAFFAPWCGHCKNMAPEYVKAETLVEKN-				82
Sbjct 16	A APE+ V+ L +F E +H +L EF+APWCGHCK +APEY KAA L +				75
Query 83	-ITLAQDCTENQDLCMEHNI PGFPSLKIFKNSDVNNSIDYEGPRTAEIVQFMIKQSQP				141
Sbjct 76	I LA+D TE DL ++ + G+P+K F+N D + +Y R A+ IV ++ K+ P				135
Query 142	AVAVVADLPAYLANETFTV----TPVIVQSGKIDAFNATFYSMANKHNDYDFVSAENAD				197
Sbjct 136	AATT---LPDGAASLVSESEVAVIGFFKDVESD-SAKQFLQAAEADIDPFQITNSND				191
Query 198	--DDFKL---SILYLPASAMDEPVVYNGKKADIADADV--FEKWLQVEALPYFGE-----ID				245
Sbjct 192	VFSKYLDKDGVLFLKFFDEG--RNNFECEVTKNLLDFIKHQLPLVIEFTQZAPKIF				249
Query 246	GSVFAQVYESGLPLGYLFYNDDEELEYKPLFTLAKKNRGLMNFVSDARKFGRHAGNL				305
Sbjct 250	GGEIKTHILLFLPKSVSYDVGK--LSNFKTA----AESFKGKILFIFIDSD-----ITDQW				299
Query 306	NK-----KEQFLFAIHDHMTDELKYLGLPQLSEAFDELSKIVLESKAIETLVKDFLX				358
Sbjct 300	RILEFFGLKKECPAVRLITLEEEMTKYKE-SEE-----LTAERITFCRHFLE				348
Query 359	GDASPIVKSQEIFENQDS--VFQVGNHDEIVNDPKDVLVLYAPWCGHCKRLAPTYQ				417
Sbjct 349	GKIFHILMSQELPEWDKQPVKVLVGNKFNDFVAFDEKKNFVYFAPWCGHCKLQFMD				408
Query 418	ELADTYANATSDVLI AKLDHTENDVRGVVIEGYPTIVLYPGGKSSSEVYQGSRLDSLF				477
Sbjct 409	+L +TY + +++IAK+D T N+V V + +PT+ +P + Y G R+LD				467
Query 478	DFIKENGH 485				
Sbjct 468	KFLSEGGQ 475				

Figure 19: BLAST search of yeast Pdi1 against the human protein database

(A) A BLAST search was performed using default parameters for yeast Pdi1 against the human protein database and the top five results indicating highest degree of similarity to Pdi1 are pictured. The alignments of yeast Pdi1 compared to the top five mammalian PDI homologs (B) ERp72, (C) PDI, and (D) ERp57 are shown.

Another criterion for this study was to ascertain which PDIs exhibited overlapping expression patterns with ApoB. PDI, ERp57, and ERp72 are all expressed in the liver (Marcus *et al.*, 1996)(<http://www.hprd.org/>), but the fourth and fifth best hits from the BLAST search, PDIP and PDILT, respectively, are not expressed in this tissue. Further, PDI, ERp57, and ERp72 have a similar domain organization to Pdi1 (a-b-b'-a'), although ERp72 has an additional thioredoxin like active site (a°-a-b-b'-a') (Hatahet and Ruddock, 2009). The crystal structures of yeast Pdi1 and mammalian ERp57 have been solved (Tian *et al.*, 2006; Tian *et al.*, 2008; Dong *et al.*, 2009) and share broad overall similarity, having domains organized in a U-shaped conformation, although ERp57 has a more twisted conformation than Pdi1 (Dong *et al.*, 2009). In ERp57 the two halves of the protein are less compact and pivot at the connection point, as compared to PDI. Interestingly, PDI and ERp72 are also able to support the growth of *pdi1Δ* yeast strains (Gunther *et al.*, 1993).

Because Pdi1's b' domain was required to facilitate ApoB ERAD in yeast (Figure 13C), I also wanted to examine mammalian PDI family members that harbor substrate binding domains. PDI, ERp57, and ERp72 all have substrate binding domains, and specific substrates have been identified for each of these proteins. The chaperone activity of PDI is necessary to refold pro-insulin (Winter *et al.*, 2002) and lysozyme (Puig and Gilbert, 1994). ERp57 specifically binds lectins through a binding site in its b' domain (Russell *et al.*, 2004) and a range of substrates have been identified for ERp57 *in vivo*, including, laminin, collagen, and clusterin (Jessop *et al.*, 2009). Similarly, ERp72 is found within chaperone complexes and interacts with thrombospondin (Kuznetsov *et al.*, 1997) and thyroglobulin (Menon *et al.*, 2007); however, it is unknown if these interactions occur through the b' substrate binding domain. Nevertheless, PDI, ERp57, and ERp72 have all previously been shown to precipitate with ApoB, and through

its integration into the MTP complex, PDI interacts with ApoB during lipid-loading ((Adeli *et al.*, 1997; Linnik and Herscovitz, 1998; Zhang and Herscovitz, 2003). However, the roles of PDI, ERp57, or ERp72 as regulators of ApoB degradation in the mammalian ER have not been examined.

In collaboration with Charles Guo in the Fisher lab at NYU Medical School, to determine whether PDI, ERp57, or ERp72 contribute to ApoB ERAD, I overexpressed each protein in rat hepatoma McA-RH7777 cells, an established cell line for studying ApoB biogenesis (Tanabe *et al.*, 1989). First, to monitor whether each protein could be overexpressed, cells were assayed after they were transfected with a vector control or vectors engineered for the transient expression of human PDI, ERp57, or ERp72. After 48 h the cells were broken and the lysates were subjected to SDS-PAGE and probed with anti-PDI, anti-ERp57, or anti-ERp72 antibodies. When duplicate samples were analyzed and the signals were quantified relative to a loading control, PDI and ERp72 were over-expressed by ~7-fold and ~6-fold, respectively (Figure 20A) and—assuming similar antibody avidity—the quantity of overexpressed ERp57 appears comparable to PDI and ERp72 when overexpressed (Figure 20A).

I hypothesized that if PDI played a strong MTP complex-independent role in mammalian cells during ApoB degradation, then its overexpression would result in decreased recovery of ApoB (Wang *et al.*, 1997a). However, if PDI's only role in ApoB biogenesis was through the MTP complex, I predicted that increased recovery of ApoB would be evident because the MTP complex promotes ApoB secretion (Gordon *et al.*, 1994; Leiper *et al.*, 1994; Haghpassand *et al.*, 1996) and escape from ERAD. No change in ApoB recovery could indicate that a pro-degradative role of PDI was balanced by the stabilizing role of the MTP complex. When the amount of radiolabeled ApoB recovered after a 60 min chase in PDI-overexpressing cells was

compared to the control, a 37% overall increase in ApoB recovery was evident (Figure 20B, left). These results suggest that the expression of greater amounts of PDI improve MTP complex function and augment ApoB transport and escape from ERAD. As controls for this experiment, Charles Guo in the Fisher lab also assessed the secretion of albumin, as described (Hrizo *et al.*, 2007; Gusarova *et al.*, 2007) but found that its biogenesis was unaffected by PDI over-expression (data not shown). In addition, Charles asked whether PDI over-expression enhanced ApoB secretion because it inhibited ERAD. However, he found that PDI over-expression, regardless of whether the proteasome was active or not (i.e., in the presence of MG-132), led to an increase in the amount of secreted ApoB (Figure 21).

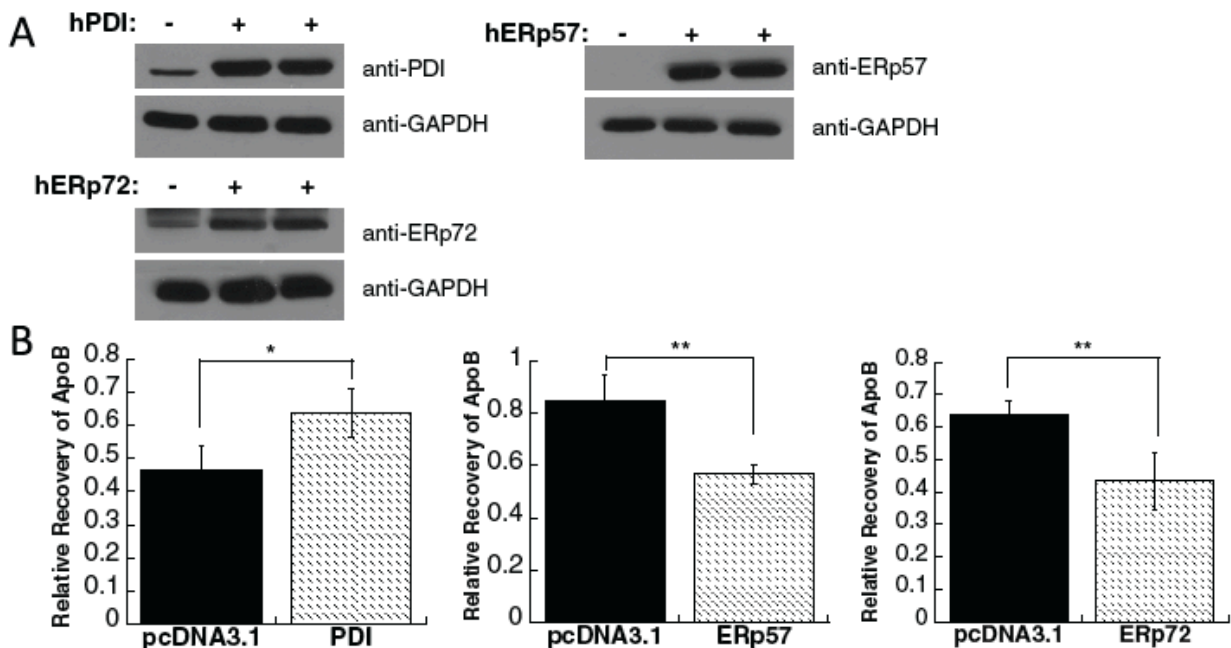


Figure 20: Over-expression of ERp57 or ERp72 leads to an increase in ApoB100 ERAD

(A) McArdle-RH7777 cells were transfected with pcDNA3.1 lacking an insert (-), or containing PDI, ERp57, or ERp72. Equal amounts of cell lysates were analyzed by immunoblotting with an anti-PDI antibody, anti-ERp57

antibody, or anti-ERp72 antibody. (B) Following a metabolic labeling reaction, a 60 min chase was performed as described in the Materials and Methods in McArdle-RH7777 cells transfected with a vector control (pcDNA3.1 lacking an insert), or containing the PDI, ERp57, or ERp72 genes. The bars indicating the “Relative Recovery of ApoB” indicate the amount of ApoB-precipitable material recovered from cell lysates and secreted into the medium at the completion of the chase divided by the amount of ApoB-precipitable material recovered after 15 min of chase. This prolonged period is required to complete the synthesis of ApoB (Gusarova *et al.*, 2001). Data represent the means of 3 experiments, \pm SEM. Where indicated (*) $p < 0.05$ and (**) $p < 0.01$.

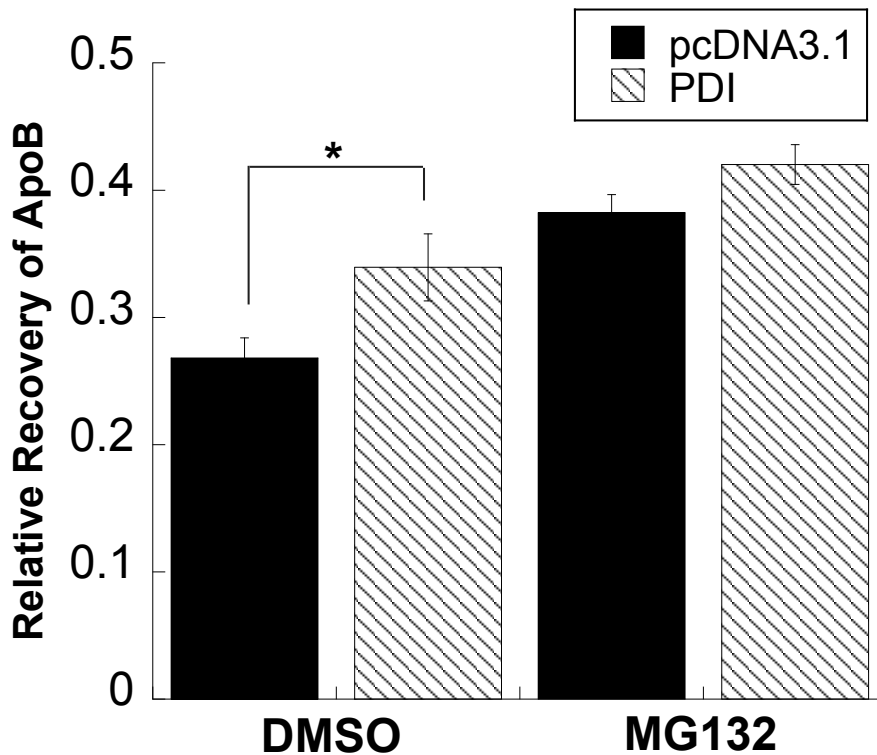


Figure 21: PDI promotes ApoB secretion independent of ERAD activity

Following a metabolic labeling reaction in the presence of either DMSO or the proteasome inhibitor MG132, as indicated, a 90 min chase was performed as described in the Materials and Methods in McArdle-RH7777 cells transfected with a vector control (pcDNA3.1 lacking an insert), or containing the PDI gene in pcDNA3.1. The “Relative Recovery of ApoB” indicates the amount of ApoB-precipitable material recovered from cell lysates and secreted into the medium at the completion of the chase divided by the amount of ApoB-precipitable material recovered after 30 min of chase. Data represent the means of 3 independent experiments, \pm SEM. Where indicated (*) $p < 0.05$.

If yeast Pdi1 is required for ApoB degradation (Figures 10 & 13), why isn't the closest homolog in human cells, PDI, similarly required for ERAD? Instead, human PDI over-expression stabilized the protein and increased its recovery in this experiment. The simplest explanation for this result is that yeast lack the MTP complex, which is required to load lipids onto maturing, ApoB-containing chylomicrons and VLDLs in the ER. In mammalian cells, the loss of MTP function—brought about either by genetic means or through the use of small molecule inhibitors—is accompanied by increased ApoB degradation (Jamil *et al.*, 1996; Benoist and Grand-Perret, 1997; Zimmermann *et al.*, 2006). Thus, our yeast ApoB model most closely resembles the lipid- or MTP complex-deficient state. I conclude that Pdi1 is a pro-degradative factor for ApoB in yeast, yet when complexed with the M subunit in mammals, the closest Pdi1 homolog, PDI, promotes ApoB folding and secretion.

Based on my yeast data, I also hypothesized that over-expression of ERp57 and ERp72 would lead to increased ApoB degradation. As predicted, when either ERp57 or ERp72 was over-expressed, ~33% less ApoB was recovered compared to the vector control (Figure 20B, middle and right panels). To confirm that ApoB degradation was proteasome-mediated and that the effect of ERp57 and ERp72 was via the ERAD pathway and not an alternate degradative system (Pan *et al.*, 2008), I assessed the impact of a proteasome inhibitor, MG132, on ApoB recovery when ERp72 was overexpressed. In this experiment, in the presence of DMSO, less ApoB was again recovered when ERp72 was overexpressed compared to the vector control, but when cells were treated with MG132, ApoB recovery in both the mock and ERp72 overexpressing cells rose (Figure 22). Overall, these data indicate that PDI is primarily an ApoB stabilizing chaperone in mammalian cells, most likely through its function as a component of the MTP complex, but that ERp57 and ERp72 facilitate ApoB ERAD (see model Figure 23).

More generally, these results support the use of the yeast ApoB expression system as a means to identify components that play diverse roles during the ERAD of this protein in mammals.

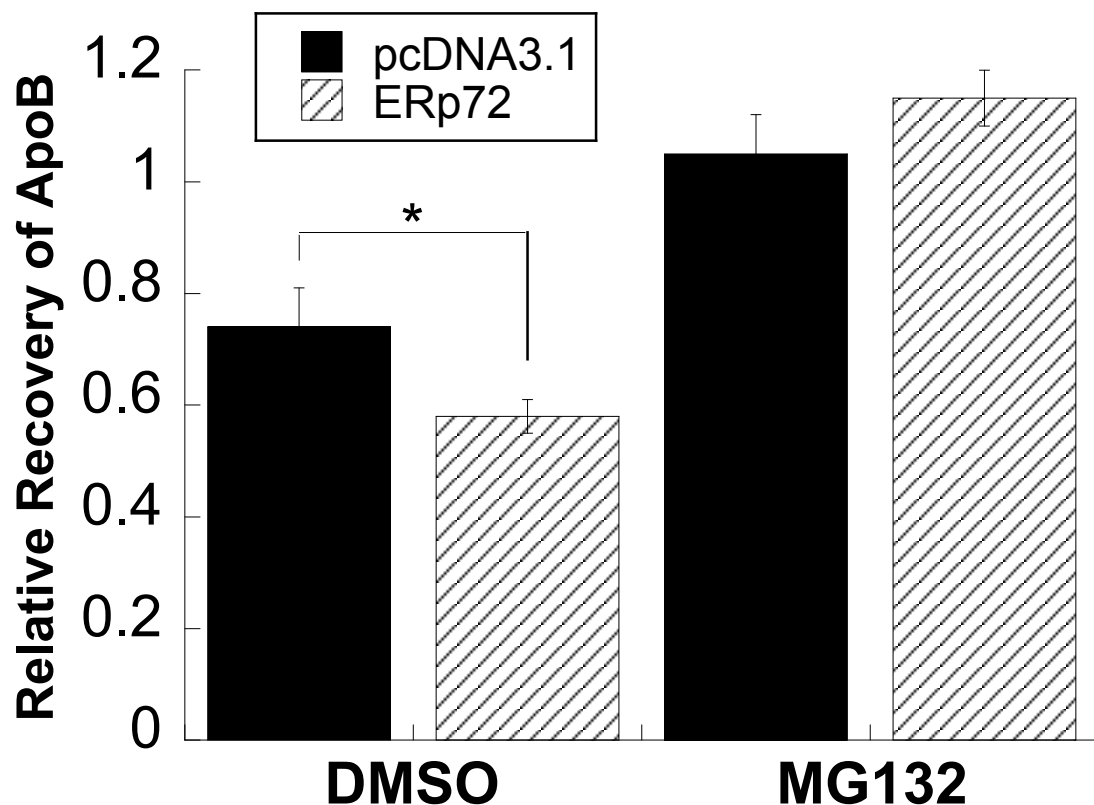


Figure 22. ApoB degradation is proteasome mediated when ERp72 is overexpressed

Following a metabolic labeling reaction in the presence of DMSO or MG132, an inhibitor of the proteasome, a 60 min chase was performed as described in the Materials and Methods in McArdle-RH7777 cells transfected with a vector control (pcDNA3.1 lacking an insert), or containing the ERp72 gene. The bars indicating the “Relative Recovery of ApoB” indicate the amount of ApoB-precipitable material recovered from cell lysates and secreted into the medium at the completion of the chase divided by the amount of ApoB-precipitable material recovered after 15 min of chase. Data represent the means of 3 experiments, \pm SEM. Where indicated (*) $p < 0.05$.

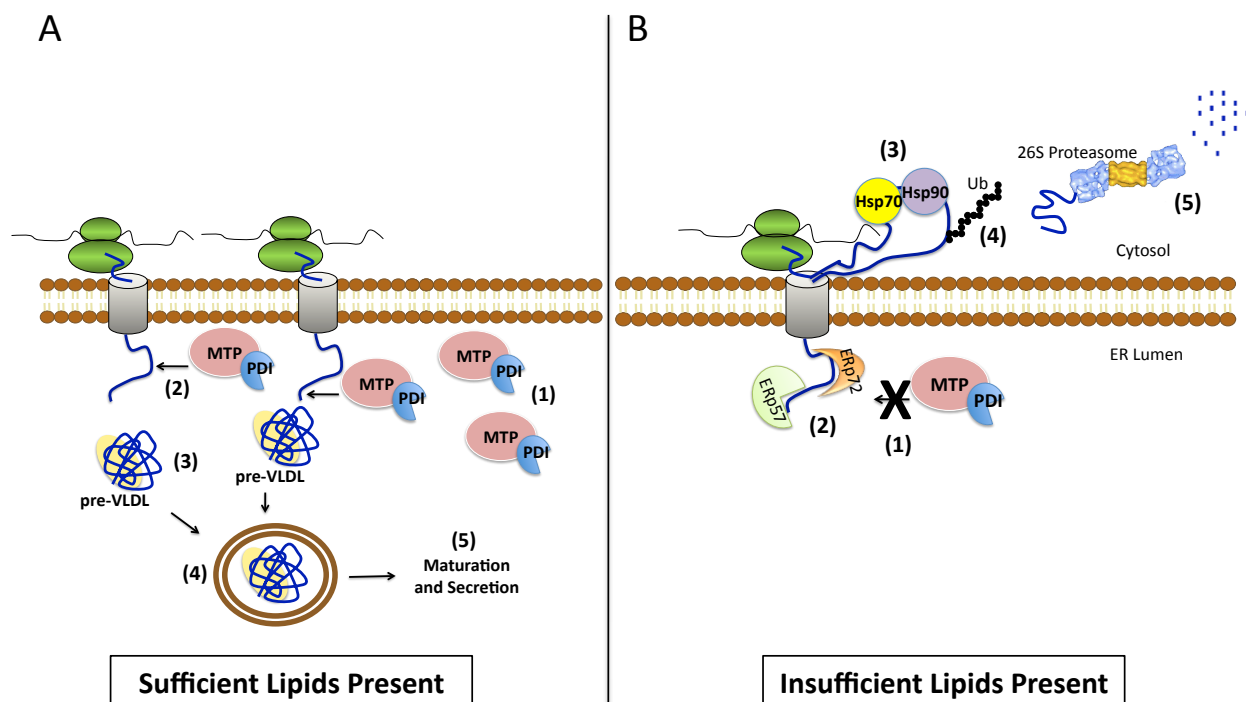


Figure 23. Model depicting the opposing roles PDI family members play in ApoB biogenesis in mammals

(A) PDI acts as a pro-stabilization and secretion factor for ApoB in mammalian cells. PDI is a subunit of the MTP complex. Its association with the M-subunit keeps the M-subunit retained and stable in the ER, suggesting that over-expression of PDI leads to an increase of the MTP complex in the ER (1). Having more MTP complex in the ER lumen allows for increased lipid loading of ApoB as the nascent protein is translated and translocated into the ER (2). Upon lipid loading, ApoB assembles into a pre-VLDL (3). The pre-VLDL is packaged into COPII vesicles (4) and trafficked to the Golgi where it undergoes further maturation into a VLDL and is then secreted into the bloodstream (5). (B) ERp57 and ERp72 are pro-degradation factors for ApoB in mammalian cells. When lipid levels are low in cells or the MTP complex is compromised, the MTP complex cannot load lipids onto ApoB as it is translated and translocated into the ER (1), leaving hydrophobic patches of ApoB exposed. To prevent aggregation of ApoB within the lumen of the ER, ERp57 and ERp72 bind to hydrophobic segments of ApoB through their chaperone binding domains (2). Since ApoB is not loaded with lipids, translocation of ApoB stalls and large cytoplasmic loops of ApoB accumulate that are recognized by molecular chaperones (3). ApoB is then poly-ubiquitinated and retrotranslocated out of the ER (4) and is ultimately degraded by the 26S proteasome (5).

2.3 DISCUSSION

In this chapter, I show for the first time that PDI family members play opposing roles during the degradation of an ERAD substrate in mammalian cells. Specifically, I found that ERp57 and ERp72 facilitate the proteasome-mediated degradation of ApoB, but PDI helps stabilize ApoB in hepatic cells, an effect most likely brought about by its membership in the MTP complex. In yeast, which lack the MTP complex, I discovered that Pdi1 facilitates the ERAD of ApoB. I also discovered that the chaperone activity of Pdi1 is required for degradation, and that in the absence of chaperone activity ApoB's conformation appears to be altered. In contrast, the degradation of another substrate (i.e., CPY*) is dependent on Pdi1's thioredoxin motifs, and yet another ERAD substrate's destruction (i.e., α -ENaC) is PDI-independent. These data highlight the complex and unique actions of PDI family members during ERAD.

Interestingly, it still remains mysterious how the generated disulfide bonds in ApoB might be broken prior to retrotranslocation. Yeast lack ERdj5, which has been proposed to perform this function in mammalian cells (Ushioda *et al.*, 2008). So, one is left with the scenario that an as-yet to be identified enzyme is capable of breaking disulfides in the yeast ER. Alternatively, the reduction of disulfide bonds may be unnecessary for Sec61-dependent retrotranslocation and proteasome degradation. It is worth noting that polypeptide "loops" can be inserted into the Sec61 translocon (Skach, 2009). Disulfide bonded substrates and circular substrates can also be degraded by the proteasome (Lee *et al.*, 2002; Liu *et al.*, 2003). In addition, large, folded domains have been shown to retrotranslocate across the ER membrane (Fiebigler *et al.*, 2002; Tirosh *et al.*, 2003; Schelhaas *et al.*, 2007).

In summary, I have shown in this chapter that members of the PDI family exhibit substrate specificity and play opposing roles in the ERAD of ApoB. In the next chapter, I

investigated factors that may mediate the interaction between the ribosome and the Sec61 translocon and facilitate the retrotranslocation and proteasomal degradation of ApoB.

3.0 THE YET PROTEINS, PUTATIVE REGULATORS OF THE SEC61 TRANSLOCON, PROMOTE APOLIPOPROTEIN B ENDOPLASMIC RETICULUM – ASSOCIATED DEGRADATION IN YEAST

3.1 INTRODUCTION

During the biogenesis of ApoB, as occurs for almost all secreted proteins, the ribosome synthesizes ApoB at the surface of the ER and ApoB is co-translationally translocated through a channel in the ER membrane, known as the Sec61 translocon, into the lumen of the ER. Once in the lumen of the ER, ApoB is loaded with lipids and assembles into a pre-VLDL, which can traffic through the secretory pathway, mature, and be secreted into the plasma (Tran *et al.*, 2002), as described in Section 1.2.2. If ApoB is not loaded with lipids, it becomes a substrate for ERAD and must be retro-translocated out of the ER into the cytosol where it is poly-ubiquitinated and degraded (Yeung *et al.*, 1996; Benoist and Grand-Perret, 1997).

In order for ApoB translocation to initiate, the ribosome must form a tight seal with Sec61, allowing ApoB entrance in to the ER without exposure to the cytosol; however, for ApoB to be retro-translocated and escape the ER lumen for degradation, the ribosome must be liberated from the ER membrane or the seal between Sec61 and the ribosome must be loosened (Beckmann *et al.*, 1997; Menetret *et al.*, 2000; Potter and Nicchitta, 2000; Beckmann *et al.*, 2001). Several candidate proteins may regulate the interaction between Sec61 and the ribosome

to facilitate the formation of a gap, which would allow ApoB exposure to the cytosol for degradation (see below).

Of the many known and characterized ERAD substrates, ApoB is the only substrate that has been shown to remain associated with the Sec61 translocon and is co-translationally degraded by the proteasome (Mitchell *et al.*, 1998; Pariyarath *et al.*, 2001; Oyadomari *et al.*, 2006). In contrast, most ERAD substrates are fully translated and translocated into the ER before selection for ERAD. As a result of ApoB's unique association with Sec61 during translocation, retrotranslocation, and degradation, I hypothesized that proteins that regulate the translocon to allow both the entrance to and exit from the ER will impact ApoB biogenesis.

Candidate yeast proteins that may regulate the Sec61-ribosome interaction include Ysy6, Ykl207w, and the YET proteins. Ysy6 is the yeast homolog of the mammalian protein RAMP4, which is suggested to be involved in the ER stress response, and is associated with both Sec61 and calnexin (Gorlich *et al.*, 1992; Schroder *et al.*, 1999; Yamaguchi *et al.*, 1999; Lee *et al.*, 2003). Calnexin has also been shown to co-precipitate with ApoB (Ou *et al.*, 1993; Patel and Grundy, 1996; Chen *et al.*, 1998; Zhang and Herscovitz, 2003). The expression of Ysy6 in *E. coli* is also sufficient to suppress a mutation in the SecY protein, the bacterial homolog of Sec61 (Sakaguchi *et al.*, 1991), suggesting that Ysy6 may aid in Sec61-mediated protein translocation. A second candidate translocon regulator is Ykl207w, which is a component of the yeast ER Membrane protein Complex (EMC). The EMC is composed of 6 proteins that genetically and physically associate with one another (Jonikas *et al.*, 2009). The Weissman lab used phenotypic interaction maps to show that the genetic stress response profiles of EMC mutants, in combination with other mutant strains, were very similar to the profiles exhibited by strains containing mutations in the translocon. Additionally, they showed that loss of the EMC

proteins leads to an accumulation of misfolded membrane proteins (Jonikas *et al.*, 2009), suggesting that the EMC may be involved in the quality control of membrane proteins. Even though ApoB is not a *bona fide* membrane protein because it has no transmembrane domains, it has been found in close association with ER microsomal membranes through its hydrophobic domains, perhaps during an initial stage of lipid recruitment (Zhou *et al.*, 2011) and, as such, may require quality control machinery utilized by membrane proteins.

Two other candidates that may act as translocon regulators are members of the YET family. The yeast proteins Yet1 and Yet3, whose human homologs are BAP29 and BAP31 respectively, are integral membrane proteins that have been proposed to aid in the biogenesis of membrane proteins (Annaert *et al.*, 1997; Schamel *et al.*, 2003; Paquet *et al.*, 2004; Ladasky *et al.*, 2006; Wilson and Barlowe, 2010). In mammalian cells, BAP31 co-precipitates with the translocon (Wang *et al.*, 2008a), and in yeast the association between Yet1 and the translocon is increased with ER stress (Wilson and Barlowe, 2010). Additionally, BAP31 has been shown to aid in the retro-translocation of an ERAD substrate, the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), and in association with Derlin to contribute to CFTR ERAD in mammalian cells (Lambert *et al.*, 2001; Wang *et al.*, 2008a). Furthermore, BAP31 promotes the escape of the SV40 virus from the ER into the cytosol during infection (Geiger *et al.*, 2011).

I hypothesized that because BAP29, BAP31, and their yeast homologs interact with the translocon and facilitate the biogenesis of membrane proteins, as well as contribute to the ERAD of another known substrate, they may facilitate ApoB retro-translocation and degradation. By using a yeast expression system for ApoB29 to initially investigate the contribution of these proteins to ApoB degradation, I found that in the absence of *YET3* or another member of the Yet family, *YET2*, ApoB is significantly stabilized, while the ERAD of two other substrates, CPY*

and $\rho\alpha f$, remains unaltered. Additionally, I found that Yet3 and ApoB coimmunoprecipitate. These preliminary results indicate that Yet3 interacts with and contributes to the degradation of ApoB. Based on my preliminary work, I have begun to investigate whether the human homolog of Yet3, BAP31 contributes to the ERAD of ApoB100 in hepatic cells.

3.2 MATERIALS AND METHODS

3.2.1 Yeast Strains

All yeast strains were grown at 26°C using standard conditions for growth, media preparation, and transformation unless otherwise noted (Adams *et al.*, 1997). A complete list of yeast strains used in this study can be found in Table 4. Strain *yet1,2,3Δ* was kindly provided by the Barlowe lab (Dartmouth College).

Table 4. Yeast strains used in the Yet study

Strain	Genotype	Source
BY4742	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	This lab
<i>yet1Δ</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yet1::KANMX</i>	Research Genetics
<i>yet2Δ</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yet2::KANMX</i>	Research Genetics
<i>yet3Δ</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yet3::KANMX</i>	Research Genetics
<i>yet1,2,3Δ</i> (CBY2357)	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yet1::KANMX yet2::KANMX yet3::KANMX</i>	Barlowe lab
<i>ysy6Δ</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ysy6::KANMX</i>	Research Genetics
<i>ykl207WΔ</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ykl207W::KANMX</i>	Research Genetics

3.2.2 Plasmids

The plasmids used in this study are shown in Table 5. To assess the degradation of ApoB29 in yeast, plasmid pSLW1-B29 was used (Hrizo *et al.*, 2007). To monitor the degradation of CPY*, I utilized pRS316CPY*-3HA, which was kindly provided by the Weissman lab (University of California, San Francisco)(Bhamidipati *et al.*, 2005). To examine the degradation of paf, plasmid pSM36-ppafΔG-HA was used (Kim *et al.*, 2005).

Table 5. Plasmids used in the Yet study

Plasmid Name	Notes	Selectable Marker	Reference
pSLW1-B29	ApoB29 expression	<i>URA3</i>	Hrizo <i>et al.</i> , 2007
pRS316CPY*-HA	CPY* expression	<i>URA3</i>	Bhamidipati <i>et al.</i> , 2005
pSM36-ppafΔG-HA	paf expression	<i>URA3</i>	Kim <i>et al.</i> , 2005

3.2.3 Cycloheximide chase analysis of ERAD substrates

The degradation of ApoB29, CPY*, and paf were assessed as described in section 2.2.3.

3.2.4 Coimmunoprecipitation Assays

Yeast cells expressing ApoB29 were immunoprecipitated using anti-HA resin and Western blot analysis was used to determine whether candidate proteins immunoprecipitated with ApoB29. Briefly, yeast cells expressing ApoB29 were grown to log phase in selectable medium containing glucose and the expression of ApoB29 was induced with galactose as described in section 2.2.3. Cells were harvested and resuspended in 10mM Tris-HCl pH7.6, 10mM EDTA, and 10% sucrose, with protease inhibitors. The cells were lysed with glass beads and were spun at 2000xg

at 4°C for 5 minutes to remove unbroken cells and any aggregated material. The supernatant was diluted 2-fold with 20mM HEPES-KOH, pH 7.4, 50mM NaCl, 1% NP-40, and protease inhibitors, and incubated overnight at 4°C with anti-HA conjugated resin (Roche) or Sepharose 6B resin (Sigma-Aldrich), as a negative control. The immunoprecipitates were washed two times with 20mM HEPES-KOH, pH 7.4, 150mM NaCl, and 0.1% NP-40 and two times with 20mM HEPES-KOH, pH 7.4, 300mM NaCl, and 0.1% NP-40 and bound proteins were eluted in sample buffer prepared with 120 mM of freshly added DTT for 5 min at 75°C for SDS-PAGE. Prior to the immunoprecipitation, 1% of the lysate was retained and loaded as a control. The samples were immunoblotted with anti-Yet3 antibody (a kind gift from C. Barlowe, Dartmouth Medical School) and the primary antibody was probed with donkey HRP-conjugated anti-rabbit IgG secondary. The Supersignal West Pico Chemiluminescent Substrate was used for detection. ApoB was detected as described above in Section 2.2.3.

3.2.5 siRNA knockdown of BAP31 in rat hepatoma cells and ApoB secretion and degradation

Rat hepatoma McA-RH7777 cells (ATCC CRL-1601) were cultured at 37°C in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 10% horse serum, L-glutamine, and penicillin/streptomycin. Cells were transfected with a range of concentrations of siRNA against BAP31 or, as a control, firefly luciferase (20nM to 100nM) using Lipofectamine 2000 transfection reagent according to the manufacturers specifications (Invitrogen). This list of siRNAs used can be found in Table 6. Effective knockdown of BAP31 was assessed by Western Blot analysis after varying lengths of time (24 – 72 hrs post transfection (hpt)). BAP31 was

detected using an anti-BAP31 (H-90) antibody (Santa Cruz Biotechnology, Inc.). The BAP31 primary antibody was decorated with donkey HRP-conjugated anti-rabbit IgG secondary antibody (GE Healthcare, Waukesha, WI). Immunoblots were also probed with an anti-beta actin (AC-15) antiserum as a loading control (Abcam, Cambridge, MA). The actin primary antibody was decorated with sheep HRP-conjugated anti-mouse IgG secondary antibody (GE Healthcare, Waukesha, WI). The Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was used for the detection of BAP31 and actin immunoblots. The signals were quantified using a Kodak 440CF Image Station and the associated Kodak 1D software (Eastman Kodak, Rochester, NY).

Table 6. siRNA sequences

Oligo Name	siRNA sequence	Source
Bap31_5	CAAGAAAUACAUGGAGGAGAAUU	Geiger et al., 2011
Bap31_6	AAAGGUGAACCUCAGAACAAUU	Geiger et al., 2011
FFL	GAAUAUUGUUGCACGAUUUUU	Weiss Lab, University of Pittsburgh School of Medicine

3.2.6 Pulse Chase Analysis of ApoB100

A pulse-chase analysis of ApoB100 was performed, as previously described (Gusarova *et al.*, 2001), after treatment with 100nM siRNA for 72 hrs. Briefly, cells were starved in medium lacking cysteine and methionine before a 15 min incubation with ³⁵S-methionine to label newly translated protein. After labeling, cells were chased with medium containing cold cysteine and methionine and medium and lysates were collected 25 min after the chase and 60 min after the

chase as an endpoint. ApoB was precipitated using as anti-ApoB antibody (Chemicon, Billerica, MA) and subjected to SDS-PAGE and phosphorimaging. The relative amount of ApoB recovered was determined by dividing the amount of ApoB present in lysates and medium at the 60 min time point divided by the amount of ApoB present in lysates at 25 min.

3.2.7 Computational Analysis

The Basic Local Alignment Search Tool or BLAST was used to compare the amino acid sequences of each of the Yet proteins to the human protein database, using the blastp algorithm and the BLOSUM62 scoring matrix using default parameters. The multiple sequence alignment algorithm T-coffee (<http://tcoffee.crg.cat/apps/tcoffee/do:regular>) was used to compare the amino acid sequence each of the Yet proteins to themselves and to the BAP proteins to determine the percent similarity and identity of each of the proteins (Notredame *et al.*, 2000).

3.3 RESULTS

3.3.1 Yet2 and Yet3 contribute to the ERAD of ApoB in yeast

In order to determine whether any of the candidate translocon regulators affect the ERAD of ApoB, I examined the stability of ApoB29 by cycloheximide chase analysis in yeast strains in which the genes encoding the potential translocon regulators were deleted (Table 4). With the

help of a rotation student, Lynley Doonan, we first measured the degradation of ApoB in yeast strains where *YSY6*, the yeast homolog of *RAMP4*, or *YKL207w*, a subunit of the EMC, were deleted. In both mutant strains we found that ApoB was degraded at wild type levels (Figure 24A and B), indicating that these proteins do not contribute to the ERAD of ApoB. I next assessed the stability of ApoB in a yeast strain where all three of the *YET* genes, which encode the yeast homologs of BAP29 and BAP31, were deleted – strain *yet1,2,3Δ*. In the absence of the Yet proteins, ApoB was significantly stabilized (Figure 24C), suggesting that one or more of these proteins facilitates the ERAD of ApoB. To determine which of the Yet proteins contribute to ApoB ERAD, I examined the degradation of ApoB in yeast strains in which each of the genes encoding these proteins had been deleted individually. When either *YET2* or *YET3* were deleted ApoB was significantly stabilized, but in the absence of *YET1* ApoB was degraded at wild type levels (Figure 24D).

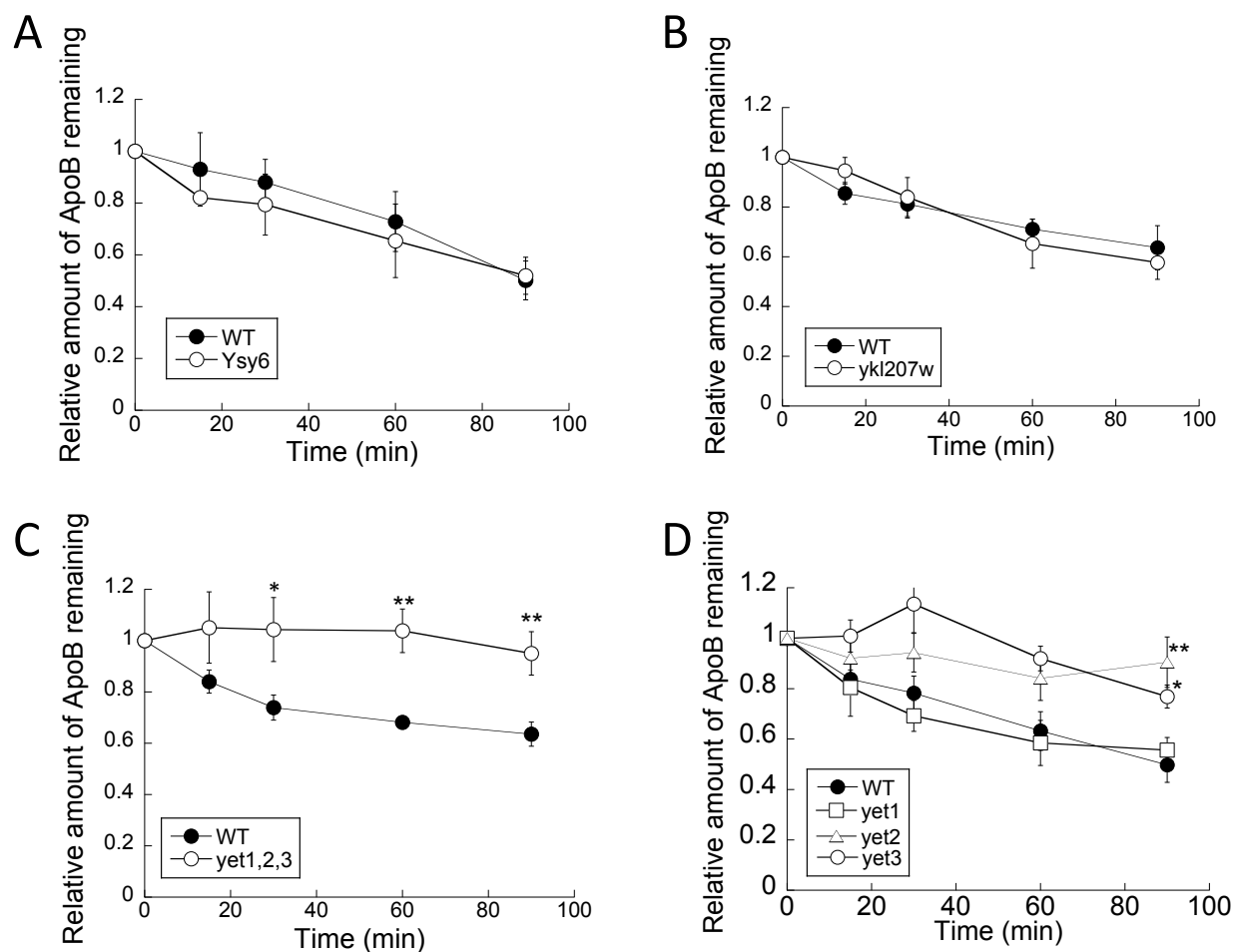


Figure 24. Yet2 and Yet3 contribute to the ERAD of ApoB

Cycloheximide chase reactions were performed as described in the Materials and Methods in WT (●), and in (A) *ysy6Δ*, (B) *ykl207wΔ*, (C) *yet1,2,3Δ*, or (D) *yet1Δ* (□), *yet2Δ* (△), and *yet3Δ* (○) yeast strains expressing ApoB29. Chase reactions were conducted at 30°C, and lysates were immunoblotted with anti-HA antibody. Anti-G6PD antiserum was used as a loading control. Data were standardized such that the “0” time point represents 100% of the protein. Data represent the means of 5-7 experiments, \pm SEM. Where indicated (**) $p < 0.01$ and (*) $p < 0.05$.

The Barlowe lab had previously reported that CPY*, a soluble ERAD substrate, and Ste6*, an integral membrane ERAD substrate, are degraded at wild type levels in the *yet1,2,3Δ* strain. They also indicated that CFTR-HA is degraded at wild type levels in the absence of *YET3*, and CPY and α -factor are translocated proficiently in the *yet1,2,3Δ* mutant (Wilson and Barlowe, 2010). In order to determine if the Yet proteins contribute to the degradation of other ERAD substrates in addition to ApoB, I measured the degradation of CPY*, to confirm or refute their results, and p α f, another soluble ERAD substrate (Caplan *et al.*, 1991), in strains deleted for either *YET2* or *YET3*. Consistent with results from the Barlowe lab (Wilson and Barlowe, 2010), CPY* and p α f were not significantly stabilized in these *YET* mutants (Figure 25 A and B). These data suggest that the Yet proteins exhibit substrate specificity during the degradation of ApoB. Based on ApoB's unique association with Sec61 during ERAD, these results suggest that the Yet proteins, which also interact with Sec61, may regulate Sec61 to facilitate the retrotranslocation and degradation of ApoB.

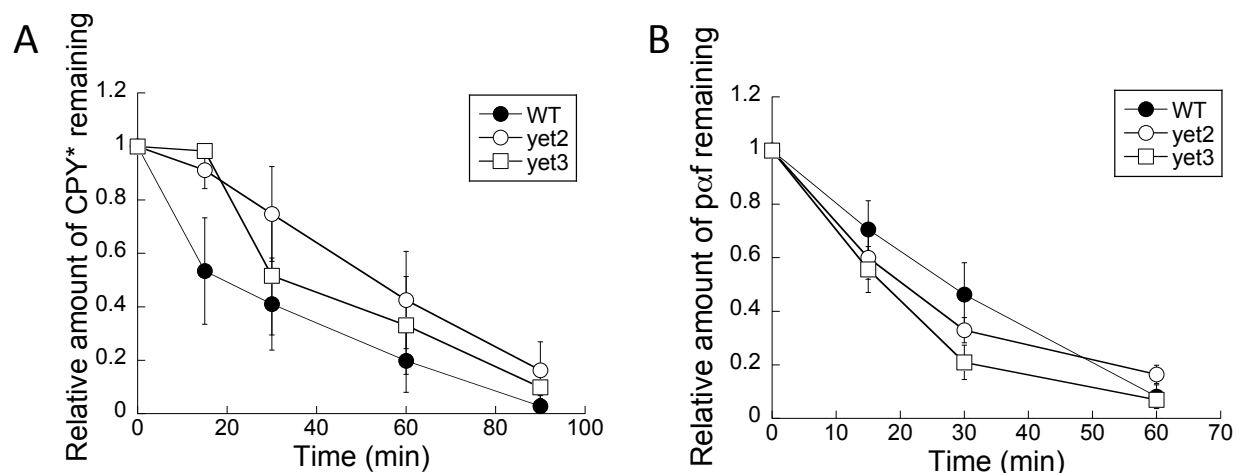


Figure 25. Yet2 and Yet3 do not contribute to the ERAD of CPY* or pαf

Cycloheximide chase reactions were performed as described in the Materials and Methods in (A) wild type (●), *yet2Δ* (○), or *yet3Δ* (□) yeast strains expressing CPY* or (B) pαf. Chase reactions were performed at 30°C, and lysates were immunoblotted with anti-HA antibody. Anti-G6PD antiserum was used as a loading control. Data were standardized such that the “0” time point represents 100% of the protein. Data represent the means of 4 experiments, ± SEM.

3.3.2 ApoB and Yet3 coimmunoprecipitate

Since ApoB was stabilized in the absence of *YET3*, I next asked whether ApoB and Yet3 coimmunoprecipitate. I found that when ApoB was immunoprecipitated with anti-HA resin Yet3 was present, but an abundant cytosolic protein, G6PD, did not coimmunoprecipitate (Figure 26). Because both ApoB and Yet3 interact with the Se61 translocon (Pariyarath *et al.*, 2001; Oyadomari *et al.*, 2006; Wilson and Barlowe, 2010), this observed interaction might not be direct. In order to confirm this interaction between ApoB and Yet3, I am currently optimizing conditions for *in vivo* cross-linking immunoprecipitation using Dithiobis[succinimidyl

propionate] (DSP) based on a protocol from the Hampton lab (Gardner *et al.*, 2000). Additionally, to better understand mechanistically how the Yet proteins contribute to ApoB ERAD, I would also like to use chemical cross-linking to investigate whether the interaction of ApoB with the degradation machinery is altered in the absence of the Yet proteins (discussed in more detail in section 4.2).

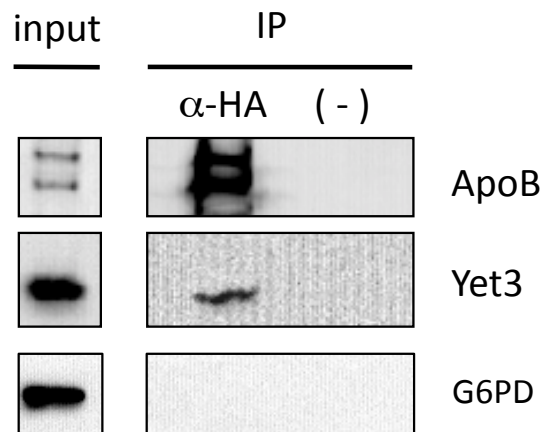


Figure 26. ApoB and Yet3 coimmunoprecipitate

Immunoprecipitation reactions were performed using anti-HA resin, or unconjugated Sepharose (“-”), using lysates from wild type yeast strains expressing ApoB29. A total of 1% of the input for the precipitation was also examined (“input”). After precipitation and SDS-PAGE, the indicated proteins were detected by immunoblot analysis.

3.3.3 BAP31 knockdown and pulse chase of ApoB in hepatic cells

To determine whether the results acquired from the use of the yeast system could be corroborated in mammalian cells, I began to examine the contribution of the mammalian Yet homolog, which may also be a potential translocon regulator, on ApoB biogenesis in hepatic cells. While there are three *YET* family members in yeast, mammals express two clear homologs, BAP29 and BAP31 (Wilson and Barlowe, 2010). In order to identify which of the BAP proteins to examine, I performed a sequence alignment of each of the yeast Yet proteins with each of the mammalian BAP proteins using the multiple sequence alignment program T-Coffee. I found that Yet3 is more similar to BAP31 than BAP29, while Yet1 and Yet2 are more similar to BAP29 (Figure 27). Because BAP31 is the closest homolog of Yet3, has been shown to promote the retro-translocation and degradation of another ERAD substrate, CFTR (Wang *et al.*, 2008a), and facilitates the dislocation of the SV40 virus from the ER to the cytosol during infection (Geiger *et al.*, 2011) I decided to initially investigate the affect of Bap31 knockdown on the biogenesis of ApoB100 in McArdle cells, which are rat hepatoma cells that are an established model for studying ApoB (see section 1.5.2). I chose not to investigate the effects of BAP31 over-expression because upon over-expression, BAP31 localizes to large aggregates, as observed by immunofluorescence, and the ER and ERAD machinery are severely disrupted (Helenius lab, personal communication).

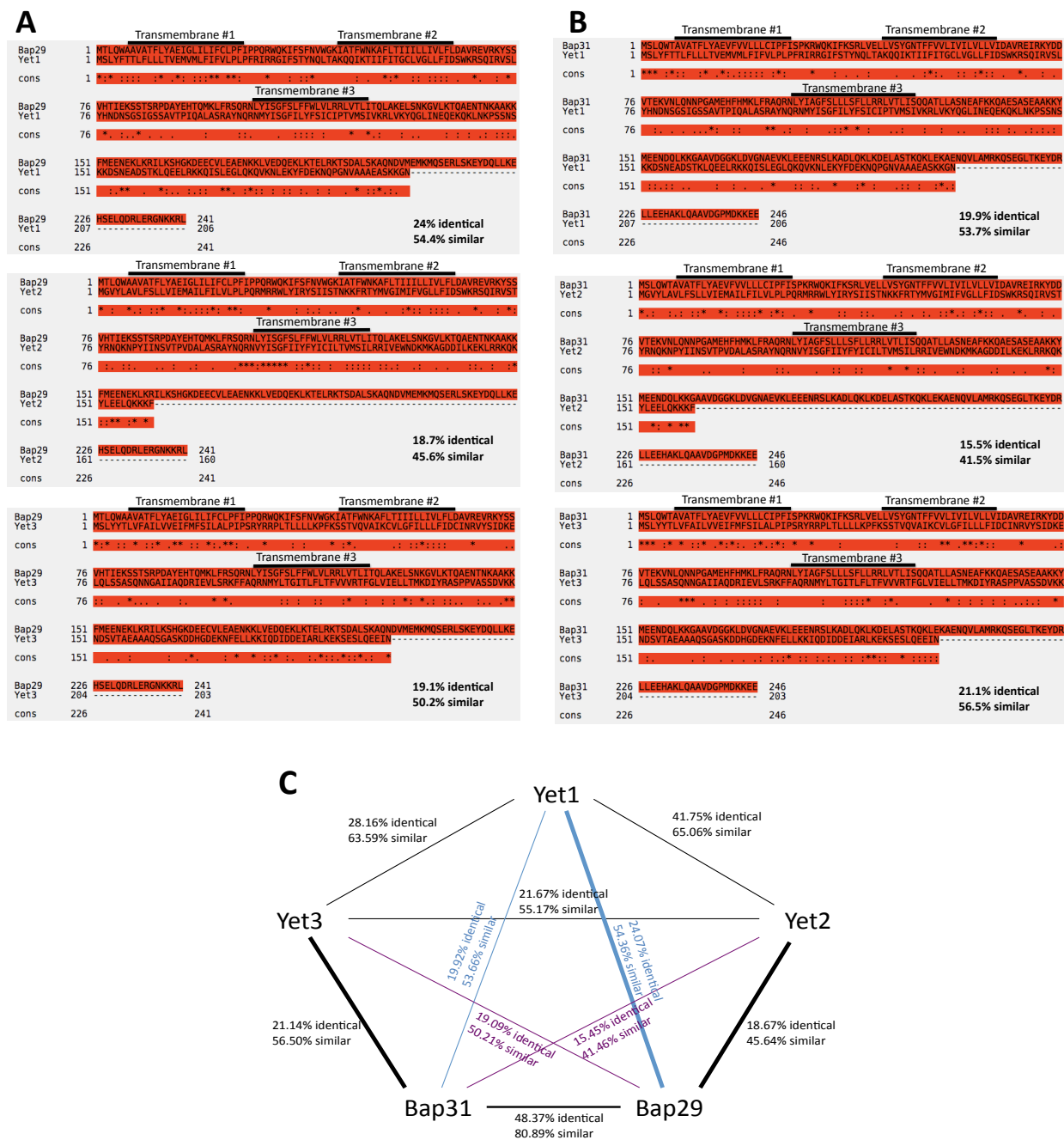


Figure 27. Percent similarity and identity between the Yet proteins and the BAP proteins

A sequence alignment of (A) Bap29 or (B) Bap31 compared to Yet1, Yet2, and Yet3 was performed using the multiple sequence alignment program T-Coffee. Identical residues are indicated with a (*) and similar residues are indicated with a (.) or (:). The transmembrane domains of the BAP proteins are shown on the alignment. Percent identity and similarity between the two proteins is reported. (C) The pentagram depicts the percent identity and similarity between each of the Yet and BAP proteins. Percent identity and similarity are recorded on the lines connecting the two compared proteins. The thicker lines indicate the BAP protein that is most similar to a particular Yet protein.

Many labs have silenced BAP31 using siRNA, and the sequences of these siRNAs have been published (Ladasky *et al.*, 2006; Regan and Laimins, 2008; Wang *et al.*, 2008a; Bartee *et al.*, 2010; Geiger *et al.*, 2011). I chose two different siRNAs, Bap31_5 and Bap31_6 (see Table 6 for sequences), which showed a high degree of knockdown in HeLa cells (Geiger *et al.*, 2011). I checked that the sequences for these siRNAs would work for rat BAP31 in addition to human, and found that the sequences were conserved with the exception of one nucleotide in siRNA Bap31_5 that I changed to fit the sequence of rat BAP31 (G to A change). In order to establish knockdown conditions for BAP31 in McArdle cells, I transfected cells with a range of concentrations of siRNA (20nM -100nM) against either BAP31 or a negative control, siRNA against firefly luciferase (FFL). I measured the amount of BAP31 protein remaining in cell lysates at 24 hpt, 48 hpt, and 72 hpt by Western blot analysis. Consistent with other reports, I did not observe knockdown of BAP31 until 72 hpt (Ladasky *et al.*, 2006; Regan and Laimins, 2008; Wang *et al.*, 2008a; Geiger *et al.*, 2011) and efficient knockdown was achieved using 100nM siRNA (Figure 28). Additionally, a double transfection of the cells 24 hrs apart did not yield additional knockdown, but caused the cells to become sick and round up (Figure 28D).

After determining that 100nM siRNA for 72 hrs provided efficient knockdown of BAP31, I wanted to repeat the transfection in quadruplicate and quantify the amount of knockdown achieved. I found that 20-30% of BAP31 protein remained after knockdown compared to the control (Figure 29), which I hypothesized would be a sufficient reduction in protein to see an effect on ApoB ERAD. Under these conditions, I performed an initial pulse chase analysis of ApoB100 to determine if ApoB ERAD was defective when BAP31 was silenced.

When the amount of radiolabeled ApoB100 recovered from cell lysates and secreted into the medium after a 60 min chase in BAP31 knockdown cells was compared to the control cells, no difference in ApoB recovery was evident (Figure 30). To determine if I would be able to observe stabilization of ApoB when ERAD was blocked, I also measured the amount of ApoB recovered from cell lysates and secreted into the medium from the control knockdown cells when the proteasome was inhibited MG-132. In this experiment, ~3.3 to 5 fold more ApoB was recovered from both cell lysates and the medium (Figure 30 – FFL-MG132 lanes). These data suggest that if knockdown of BAP31 did prevent ApoB ERAD I would have observed increased ApoB100 recovery.

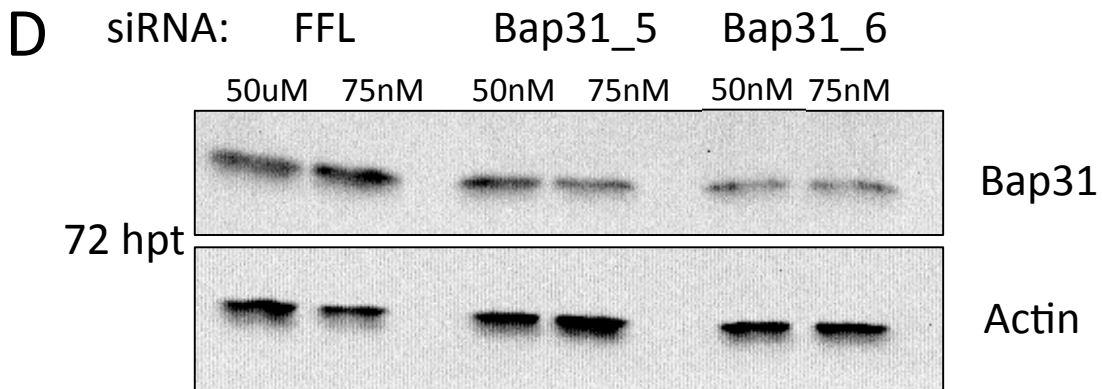
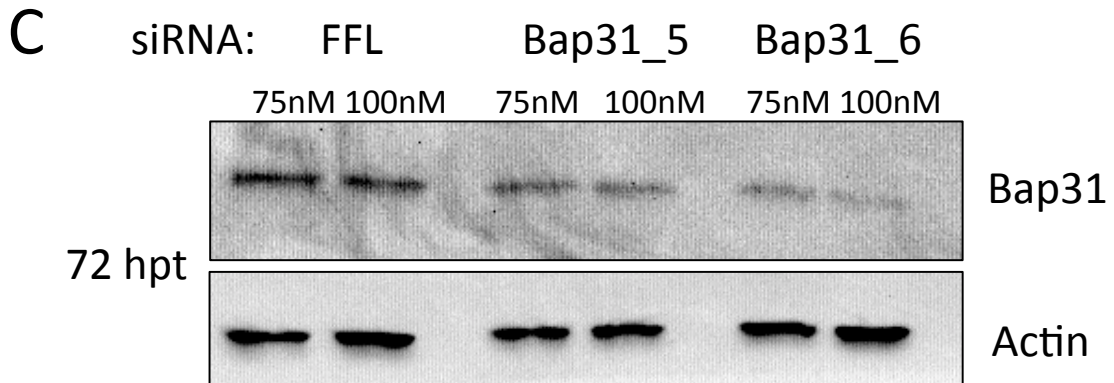
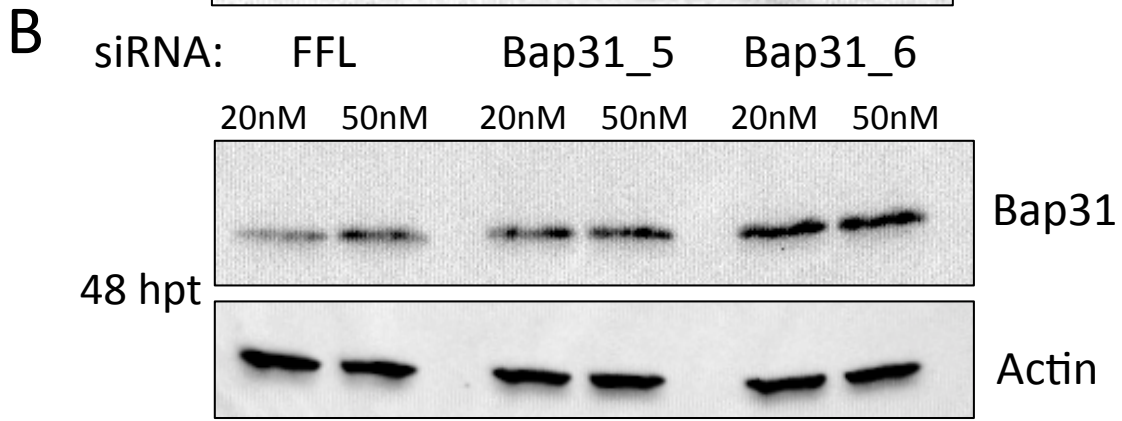
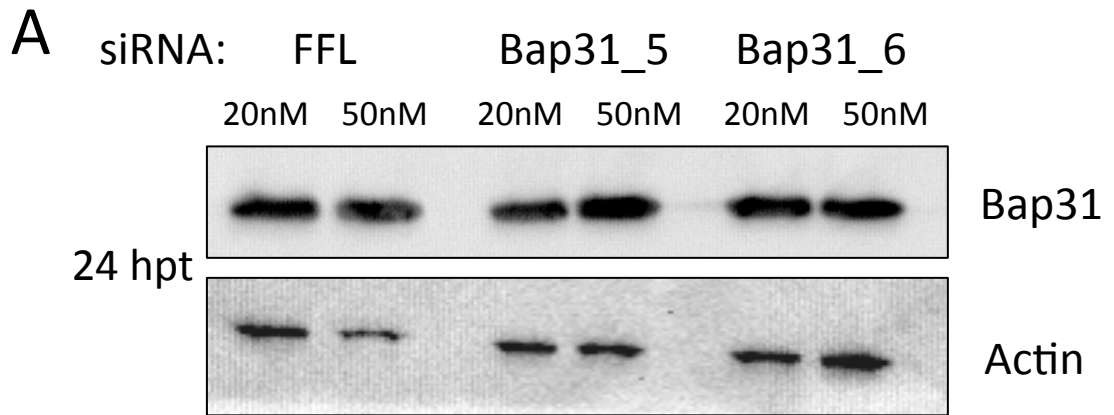


Figure 28. Establishing knockdown conditions for BAP31 in McArdle cells

McArdle cells were transfected with a range of siRNA concentrations against BAP31 (Bap31_5 and Bap31_6) or, as a control, Firefly Luciferase (FFL) (20nM to 100nM) using Lipofectamine 2000. Effective knockdown of BAP31 was assessed by Western Blot analysis (A) 24 hrs post transfection (hpt), (B) 48 hpt, or (C) 72 hpt using an antibody against BAP31. Anti-beta actin antiserum was used as a loading control. For (D), cells were transfected with a range of siRNA concentrations, as in (A,B,and C), but 24 hrs after the first transfection, the cells were again transfected with the same concentration of siRNA. Effective knockdown of BAP31 was assessed by Western Blot analysis 72 hrs after the second transfection using an antibody against BAP31. Anti-beta actin antiserum was used as a loading control.

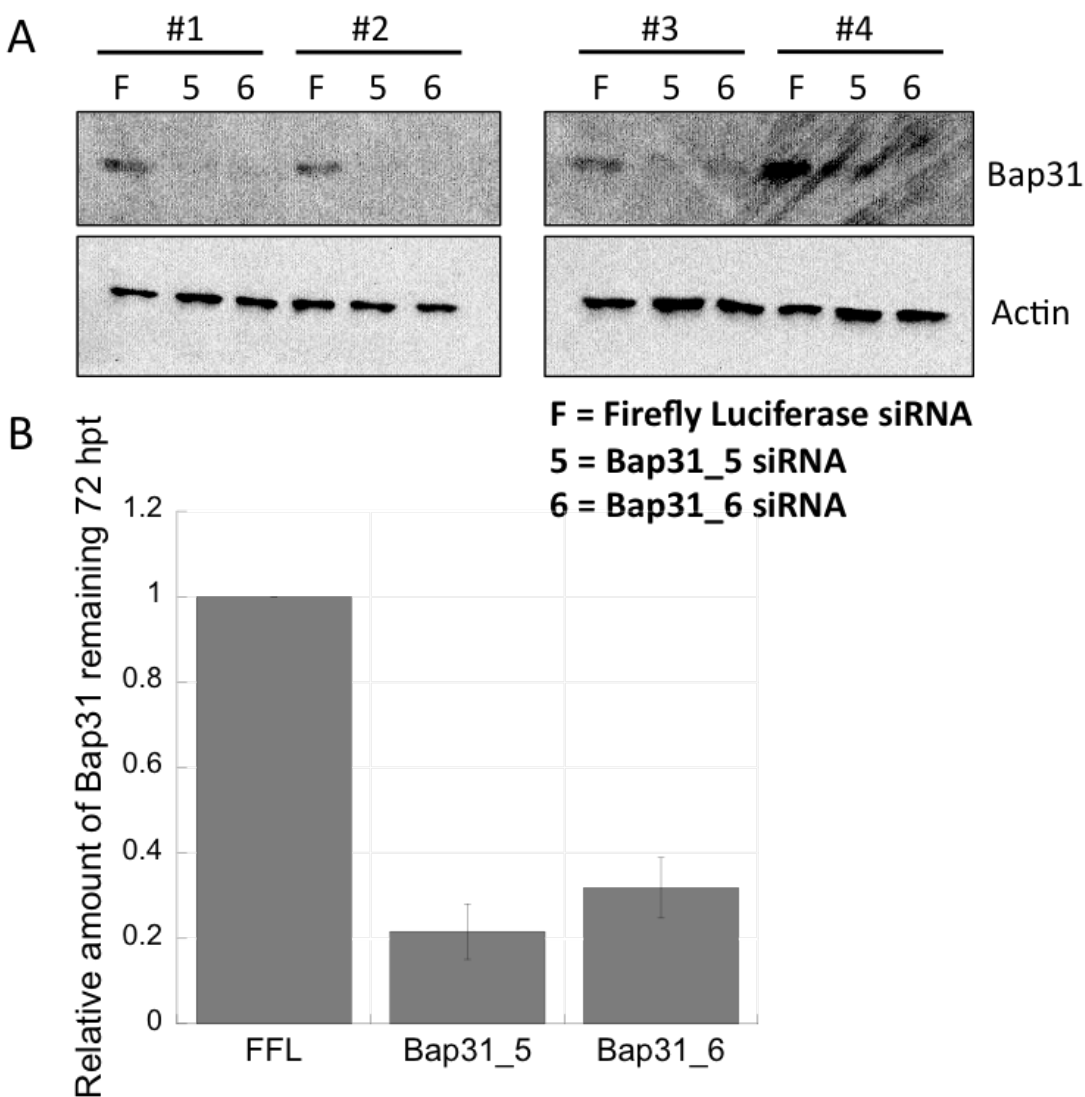


Figure 29. BAP31 protein is reduced 72hpt using 100nM siRNA

McArdle cells were transfected in quadruplicate with 100nM of siRNA against BAP31 (Bap31_5 and Bap31_6) or, as a control, Firefly Luciferase (FFL) using Lipofectamine 2000. (A) Effective knockdown of Bap31 was assessed by Western Blot analysis 72 hpt using an antibody against BAP31. Anti-beta actin antiserum was used as a loading control. (B) A quantification of the relative amount of BAP31 protein remaining 72 hpt standardized to the FFL control \pm SEM is shown.

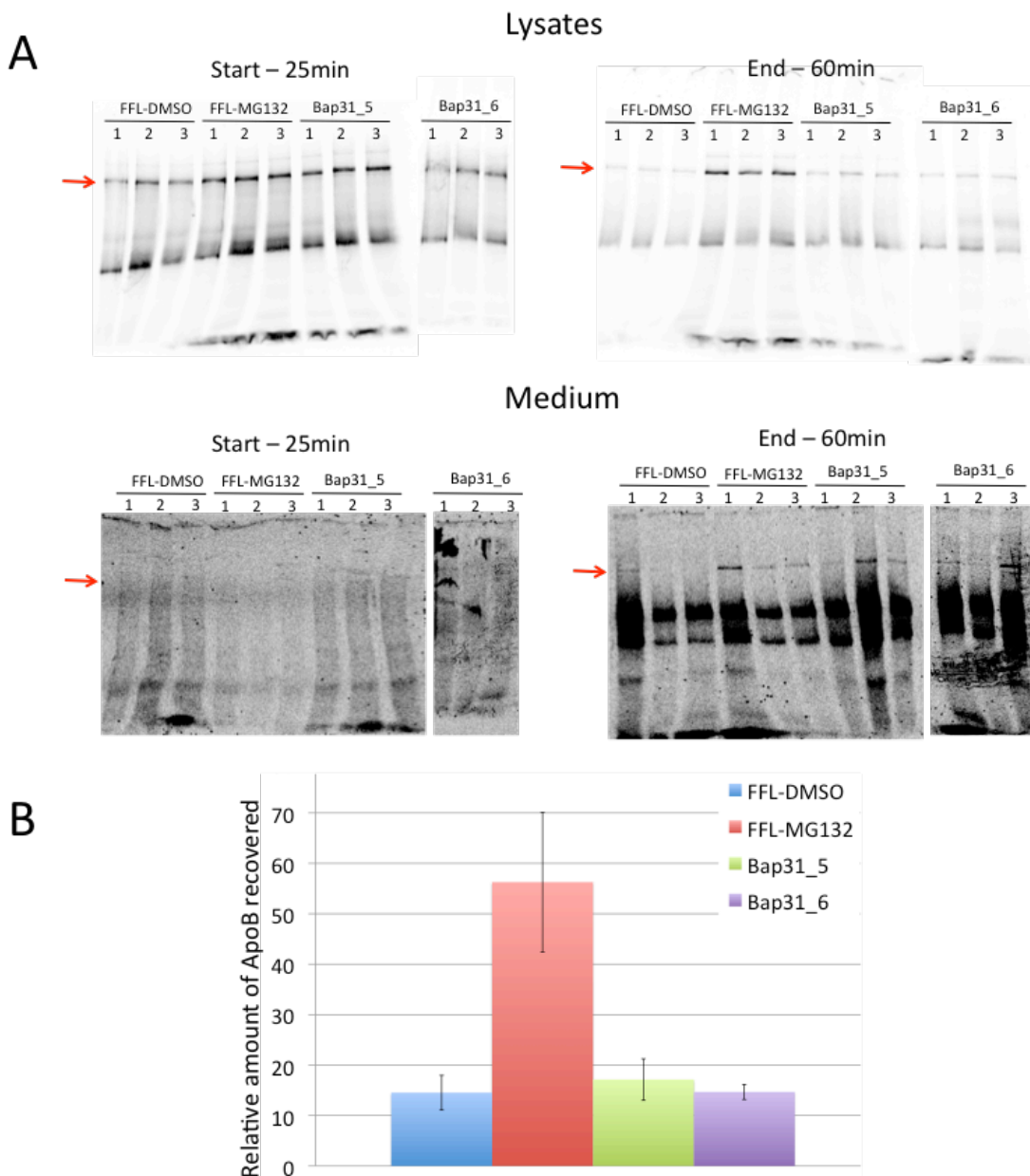


Figure 30. Proteasome inhibition leads to increased recovery of ApoB100

Following a metabolic labeling reaction, a 60 min chase was performed as described in the Materials and Methods (section 3.1.6) in McArdle-RH7777 cells transfected in triplicate with 100nM of a control siRNA (FFL), or siRNAs against Bap31 (Bap31_5 or Bap31_6) for 72 hrs. During the pulse chase the FFL transfected cells were treated with either DMSO, as a control, or 25 μ M MG132, to inhibit the proteasome. (A) The image represents ApoB-precipitated material subjected to SDS-PAGE and phosphorimaging. The red arrow indicates the band corresponding to ApoB100. (B) The “Relative Recovery of ApoB” is shown under the indicated conditions. Data

were standardized by measuring the amount of ApoB precipitated material recovered from cell lysates and secreted into the medium at the completion of the chase divided by the amount of ApoB-precipitable material recovered after 25 min of chase, i.e. the total ApoB pool. Data represent the means of 3 experiments, \pm SEM.

The results presented in Figure 30 can be explained by several possible scenarios. The first scenario is that although I observed stabilization of ApoB in yeast in the absence of Yet3, which is most similar to BAP31, in mammalian cells there may be some compensation from BAP29, since BAP29 and BAP31 share such a relatively high degree of similarity (Figure 27). Indeed, since I also observed stabilization of ApoB in the absence of Yet2, which is most similar BAP29, BAP29 may also contribute to ApoB ERAD. A second possibility is that the transfection and knockdown may not have been efficient in this experiment. Although I established consistent knockdown conditions for Bap31, in the interim between experiments our lab tissue culture room was moved to a new facility and a new vial of cells was used to continue the experiments. A third explanation is that there are differences in the regulation of ApoB29 vs ApoB100 with respect to translocation and retrotranslocation. Shorter ApoB isoforms may be differentially selected for ERAD and I could examine both ApoB29 and ApoB48 degradation in McArdle cells as well as ApoB100. Another possibility is that BAP31 acts during early events that are missed in this experiment. If BAP31 facilitates the retrotranslocation of ApoB very early as it is being translocated, I may have missed differences in degradation between control and knockdown cells because at later time-points another quality control pathway such as PERPP may take over. To alleviate this possibility I can take earlier time-points (starting at 12 min when the S³⁵ has been incorporated into translating ApoB) to ensure that I do not miss a population of ApoB that will be degraded early in its synthesis. In any event, to determine whether BAP31 had been efficiently depleted from the cells used in the pulse chase experiment, I performed a Western blot analysis of the lysates. Although I had previously observed knockdown of BAP31 at 72 hpt with 100nM siRNA, in this experiment BAP31 protein levels were not significantly reduced in the knockdown samples (Figure 31). The lack of knockdown

most likely explains why I failed to observe a difference in the amount of ApoB recovered in “knockdown” cells vs control cells. Needless to say, these experiments will be repeated after knockdown of BAP31 is verified.

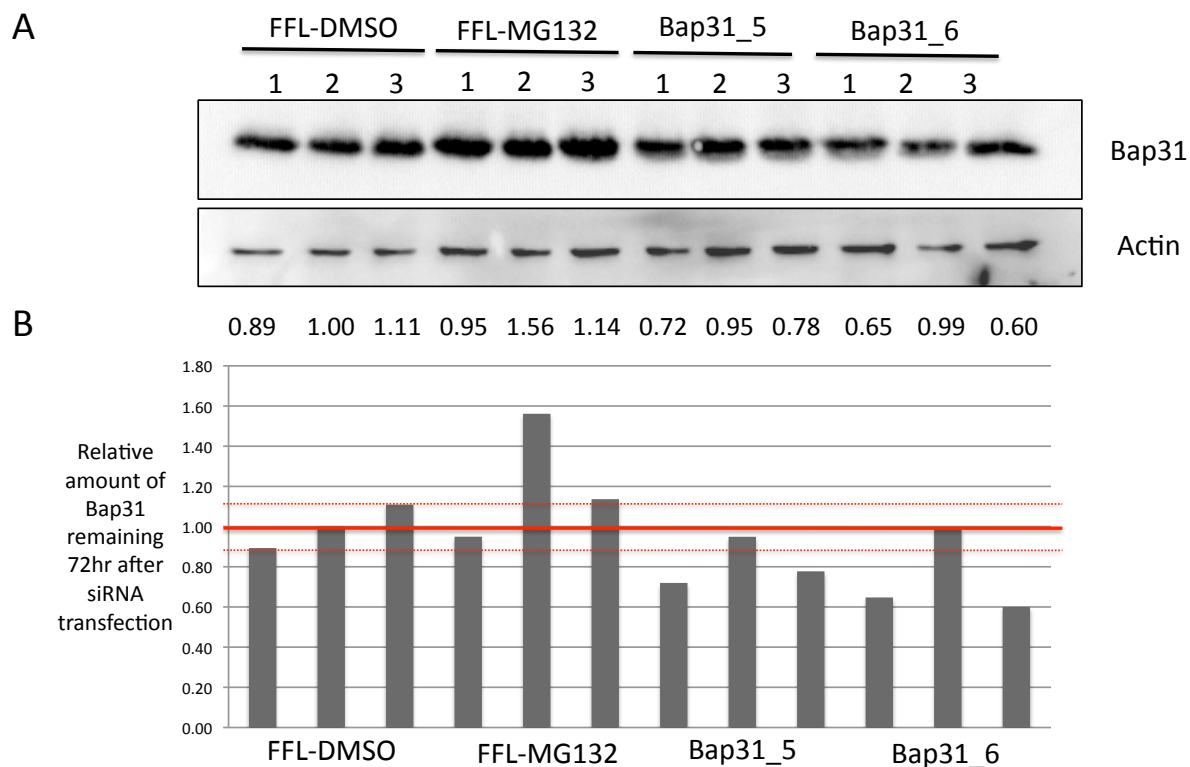


Figure 31. Bap31 was not efficiently knocked down in the cells used for the pulse chase experiment shown in Figure 30

Cell lysates from the pulse chase experiment in Figure 30 were subjected to SDS-PAGE and Western blot analysis to determine if BAP31 had efficiently been knocked down. (A) The Western blots were probed with an anti-BAP31 antibody or an anti-beta actin antibody as a loading control. (B) A quantification of the relative amount of BAP31 protein remaining 72 hpt compared to the control FFL_DMSO cells, with the numerical values listed above the graph is shown. The solid red line indicates 100% of BAP31 protein remaining and the dashed red lines indicate standard deviation of the mean from the FFL-DMSO samples.

3.4 DISCUSSION

In this chapter I showed first that two potential translocon regulators Ysy6 and Ykl207w did not impact ApoB ERAD. In contrast, two members of the Yet family of proteins, Yet2 and Yet3, contribute to the degradation of ApoB29 in yeast. Among the substrates that we and other labs have investigated, the impact of Yet2 and Yet3 on ERAD was unique for ApoB. I also began to investigate whether the mammalian homolog of Yet3, BAP31, contributes to the ERAD of ApoB100 in rat hepatoma cells. Thus far, I have developed silencing conditions for BAP31, but still need to work on consistent knockdown. Together, the results in this chapter suggest that the unique attributes of ApoB, namely the continued association with Sec61 during ERAD, lead to the specific requirement of the Yet proteins for ApoB in yeast.

One surprising aspect of this study was that while Yet2 and Yet3 were necessary for ApoB ERAD, Yet1 was not essential. This was surprising because the Barlowe lab has been unable to phenotypically separate the function of Yet1 and Yet3 and they suggest that these proteins act as a Yet1-Yet3 complex (Wilson and Barlowe, 2010). However, the phenotype that they were observing was growth sensitivity to inositol starvation, which may not be as sensitive of an assay as a degradation assay or the requirement for Yet1 and Yet3 may be different for ERAD than for growth without inositol. Interestingly, deletion of *YET3* is more disruptive than deletion of *YET1* such that in a *yet3Δ* mutant most of the Yet1 protein is depleted whereas in a *yet1Δ* mutant the majority of the Yet3 protein remains stable (Wilson and Barlowe, 2010). This could indicate that there is some redundancy among these proteins. ApoB is significantly stabilized when *YET3* is deleted and, as described above, under these conditions, Yet1 protein levels are drastically reduced. This data suggests that one or both of these proteins contributes to ApoB ERAD. However, when *YET1* is deleted there is still a sufficient amount of Yet3 protein

present. If Yet1 and Yet3 have redundant functions in ERAD, in a *yet1Δ* mutant Yet3 is still present and sufficient to function in promoting ApoB degradation, so no defect in ERAD is observed.

A second confusing result that I observed was the lack of knockdown of BAP31 when I did the pulse chase analysis of ApoB. One reason for the lack of knockdown is that when I thawed a new vial of cells, the new cells were stressed, perhaps as a result of dehydration from being frozen too quickly and did not transfect well. To test this hypothesis, I have currently broken out a new aliquot of cells and I will transfect them with siRNA and test for knockdown before doing a pulse chase experiment. A second explanation for the lack of knockdown is that my siRNA aliquots have become unstable from repeated freeze/thaw in our -20°C freezer and is no longer functioning. I can verify the concentration of the siRNAs using a UV spectrophotometer at 260nm. If I still do not observe knockdown using new cells, I will order fresh siRNAs to continue these experiments. It is also possible that even after efficient silencing of BAP31 the ERAD of ApoB will remain unaltered in McArdle cells. In this case I will try to knockdown both BAP31 and BAP29 to avoid possible redundancy and compensation. Additionally, when I repeat the pulse chase experiments I will examine other isoforms of ApoB (i.e. ApoB48) as well as examine earlier time-points within the chase to avoid missing any early ERAD decisions.

Overall, my yeast data suggest that the Yet proteins facilitate ApoB retrotranslocation and degradation (for a model see Figure 32). Using hepatic cells, I hope to recapitulate and extend these findings.

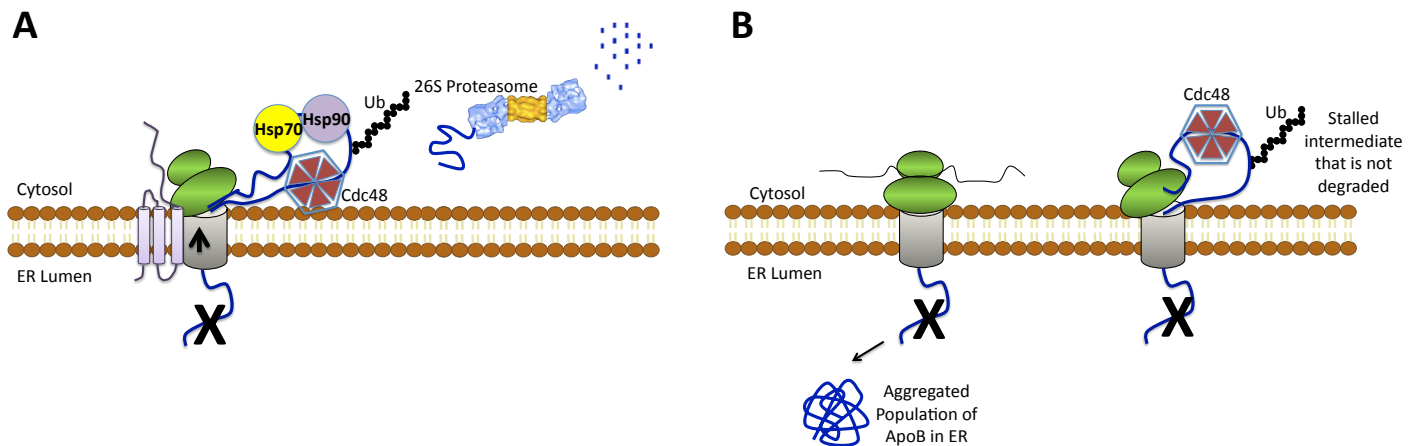


Figure 32. The Yet proteins in yeast facilitate ApoB retrotranslocation and degradation

(A) In yeast, lipids cannot be loaded onto ApoB and it is selected for ERAD. The Yet proteins may facilitate the loosening of the seal between the ribosome and Sec61 allowing ApoB to be retrotranslocated out of the ER. Loops of ApoB accumulate in the cytosol that are recognized by Hsp70 and Hsp90, and the AAA-ATPase Cdc48. ApoB is polyubiquitinated, and degraded by the 26S proteasome. (B) In the absence of the Yet proteins, when lipids cannot be loaded onto ApoB, the seal between the ribosome and Sec61 may remain intact allowing ApoB to be fully translated and translocated into the ER. This population of ApoB may remain stable in the ER in a conformation that does not allow for retrotranslocation. Alternatively, ApoB may be partially retro-translocated in the absence of the Yet proteins and may remain associated with Cdc48 as a stalled intermediate that cannot be degraded by ERAD.

4.0 DISCUSSION AND CONCLUSIONS

ApoB is targeted for degradation unlike any other known ERAD substrate. Under lipid-poor conditions in mammalian cells, ApoB is cotranslationally selected for degradation by the Hsp70 and Hsp90 molecular chaperones, as well as a J domain-containing co-chaperone, P58^{IPK}. The apolipoprotein is then ubiquitinated by gp78 while translocon-embedded and is retrotranslocated into the cytosol through the Sec61 translocon by the action of the AAA-ATPase, p97. Ultimately, this immature form of ApoB is captured and degraded by the proteasome. Given these unique attributes, and because of the profound link between ApoB secretion and human disease, I sought to identify and characterize other factors that impact the decision between stabilizing and degrading ApoB, as described in the previous chapters. I found that two members of the PDI family, ERp57 and ERp72 promote the degradation of ApoB, while another PDI family member, PDI facilitates ApoB lipid loading and secretion. These studies were first made possible by the use of a yeast expression system in which analogous PDIs facilitated ApoB29 degradation. Additionally, I discovered that two members of the yeast Yet family of proteins contribute to ApoB degradation and have begun to investigate the role of the mammalian Yet homologs using a rat hepatoma cell line. Overall, I hope that these ongoing studies will provide therapeutic candidates that may be targeted to prevent the catastrophic effects of diseases related to ApoB-containing lipoproteins, in particular atherosclerosis, which is the leading cause of death in western societies.

4.1 PDI FAMILY MEMBERS HAVE DIFFERENTIAL EFFECTS ON THE BIOGENESIS OF APOLIPOPROTEIN B

ApoB maturation requires the formation of specific disulfide bonds, and mutations in cysteines that form disulfide bonds in ApoB diminish VLDL assembly and secretion (Huang and Shelness, 1997; Tran *et al.*, 1998; DeLozier *et al.*, 2001). Mutations in the M subunit of the MTP complex, which contains PDI, can also prevent ApoB maturation, a phenomena that results in abetalipoproteinemia (Narcisi *et al.*, 1995; Ohashi *et al.*, 2000). Because I observed measureable effects of PDI, ERp57 and ERp72 over-expression on the amount of secreted ApoB, my results may be relevant to explain the relative severity of human diseases such as hypobetalipoproteinemia and atherosclerosis. In most cases, the factor(s) that lead to differences in the circulating lipoprotein levels in the population are mysterious. Polymorphisms in ApoB itself are known to contribute to altered levels of secreted VLDLs, as specific non-sense codons in *APOB* cause hypobetalipoproteinemia (Linton *et al.*, 1993; Whitfield *et al.*, 2004). Therefore, it is plausible that variations in the amount or activities of components that control ApoB maturation, such as PDI family members, may be at-play in the development of human diseases such as hypobetalipoproteinemia or atherosclerosis.

Using the yeast expression system, I found that the ERAD of ApoB required Pdi1, and more specifically, the chaperone activity provided by the b' domain of the protein. When identifying candidate mammalian PDI proteins to investigate, one of the criteria was the presence of a b' domain. Erp57 and ERp72 both have b' substrate binding domains; however, I did not

investigate whether these domains were necessary for promoting the ERAD of ApoB. My working model is that when lipids are limiting, ERp57 and ERp72 bind to ApoB through their substrate binding domains and help to keep the protein from aggregating in the ER lumen so that ApoB can be retrotranslocated and degraded.

In order to more mechanistically understand the contribution of these proteins to ApoB degradation, it would be interesting to make over-expression constructs of both ERp57 and ERp72 with various mutations. I hypothesize that if the b' domain of ERp57 or ERp72 was either deleted or residues in the binding pocket were mutated to non-hydrophobic residues and the proteins were over-expressed they would be unable to promote the ERAD of ApoB, as observed for the wild type proteins. This hypothesis could also be investigated by over-expressing the b' domain mutants in ERp57 or ERp72 knockout cell lines to determine if ApoB degradation is restored. Although ERp57 null mice are embryonic lethal (Coe *et al.*, 2010), ERp57^{-/-} and ERp72^{-/-} MEF cell lines are available (Zhang *et al.*, 2009). However, a defect in ApoB degradation when using b' domain mutants does not rule out the possible involvement of the active site domains of these proteins in ApoB degradation. The b' substrate-binding domain may be necessary for PDI to interact with ApoB, which would bring the active sites in close enough proximity to catalyze redox reactions. To determine whether the active site functions are necessary for ApoB ERAD, I could over-express ERp57 or ERp72 constructs with Cys to Ser mutations in each of the active sites individually and in tandem and determine if these proteins still contribute to ApoB ERAD.

In my model, ERp57 and ERp72 bind to ApoB through their b' domains because ApoB has highly hydrophobic patches that need to be protected from aggregation. Alternatively, ERp57 may interact with ApoB because it is a highly glycosylated protein, as are the majority of

ERp57's client proteins (Jessop *et al.*, 2009). One limitation of the yeast ApoB expression system is that of the 16 N-linked glycosylation sites utilized in ApoB100, only 2 are present in ApoB29 (Harazono *et al.*, 2005). Although I have not been able to confirm that they are glycosylated in yeast, but the Hochstrasser lab did observe ApoB29 glycosylation (Rubenstein *et al.*, 2012). To address whether the glycosylation status of ApoB influences the requirement of ERp57 for ApoB ERAD, I could examine the degradation of versions of ApoB with asparagine to glutamine mutations, which would eliminate glycosylation sites, when ERp57 is overexpressed or knocked down.

In yeast, I was able to rule out the possibility that the stabilization of ApoB29 that I observed in the absence of Pdi1 function was an indirect effect of the need for Htm1, the yeast homolog of EDEM. EDEM is an α -mannosidase like protein that interacts with glycosylated proteins and targets them for degradation. However, this does not eliminate the possibility that EDEM is involved in targeting ApoB for ERAD in mammalian cells because of the differences in ApoB glycosylation between the two systems. In fact, it would be interesting to investigate whether EDEM contributes to ApoB ERAD in hepatic cells. To address this question EDEM could be overexpressed or silenced, both of which have previously been done (Hosokawa *et al.*, 2006; Kosmaoglou *et al.*, 2009) and the ERAD of ApoB could be investigated.

Another open question is whether the EDEM associated PDI family member ERdj5 reduces the disulfide bonds of ApoB prior to retrotranslocation. Are ApoB's disulfide bonds oxidized before the ERAD decision is made? One possibility is that even if disulfide bonds in ApoB are formed before ApoB is targeted for degradation, they do not need to be reduced for the protein to escape the ER and be degraded (Fiebiger *et al.*, 2002; Lee *et al.*, 2002; Liu *et al.*, 2003; Tirosh *et al.*, 2003; Skach, 2009). To determine whether ApoB disulfide bonds are oxidized

before ERAD, a pulse chase reaction combined with a cysteine modification assay using PEG-maleimide (see section 2.2.4) to examine the timing of oxidation compared to the timing of degradation could be performed. Additionally, a co-immunoprecipitation of ApoB and ERdj5 would provide insight as to whether the two proteins interact.

If the b' domains of ERp57 and ERp72 are necessary for ApoB ERAD because of ApoB's hydrophobic character, another candidate PDI family member to investigate is ERp44. ERp44 has an a-b-b' domain organization and has been shown to regulate the IP3 receptor (Higo *et al.*, 2005) and to interact with adiponectin (Wang *et al.*, 2008b), which is a hormone that regulates fatty acid oxidation. Interestingly, when a BLAST search was performed using only the b' domain of yeast Pdi1 compared to the human protein database the only homologous protein returned was ERp44 (Hatahet and Ruddock, 2009). The b' substrate-binding domain of ERp44 may be important to maintain ApoB solubility in the ER lumen. Because ERp44 cannot act as an oxido-reductase due to its CRFS active site sequence (although it could act as an isomerase), the question of redox activity vs chaperone activity for the protein's involvement in ApoB ERAD would not be an issue. Importantly, ERp44 is expressed highly in the liver (Long *et al.*, 2011).

Along with my discovery that select PDI family members contribute to ApoB ERAD, I also found instead that the over-expression of PDI itself promoted ApoB secretion. Consistent with this finding, mice deficient for a key regulator of the unfolded protein response, Ire1, that was specifically deleted in their hepatocytes (Ire1 $\alpha^{-/-}$) (Zhang *et al.*, 2011) show impaired VLDL assembly in the ER (Wang *et al.*, 2012). The defect in VLDL assembly resulted from a reduction in MTP activity because PDI expression was decreased (Wang *et al.*, 2012). Based on these results, a remaining question is whether the interaction of PDI with MTP directly promotes

ApoB secretion or whether PDI active sites contribute to ApoB folding, and promote secretion as well. To address this question, I could create Cys to Ser active site mutants in PDI, leaving the PDI/M subunit interaction domain intact and over-express the construct in McArdle cells to determine if this mutant form of PDI facilitates ApoB secretion.

Another limitation of the yeast expression system is that we can only investigate one arm of ApoB biogenesis – ERAD, not maturation and secretion. In order to expand the yeast ApoB29 expression system, I would like to express the M subunit of the MTP complex in yeast and determine if it associates with Pdi1 to form an active complex. To avoid having to express multiple plasmids in yeast, a galactose inducible bi-directional expression vector (pBEVY) (Miller *et al.*, 1998) could be used to express both ApoB29 and M at the same time and in the same yeast cells. Under these conditions would ApoB become lipid loaded? This question could be answered using sucrose gradient floatation assays and measuring the density of the ApoB particles. Additionally, oleic acid could be added to the medium to promote lipid loading. Even if ApoB was lipid loaded by the MTP complex it may not be secreted, therefore one would need to investigate where the lipid modified protein localizes in the cell or if it becomes degraded. Under these conditions, another quality control pathway, such as PERPP, may recognize the foreign protein and degrade it. Could we use this system to study factors involved in secretion? Potentially this system could be quite useful in dissecting the secretion pathway for ApoB, as a whole genomic screen for secretion defects of specific proteins is quite easily performed using yeast (Gelling *et al.*, 2012). Finally, because Pdi1 facilitated ApoB degradation in yeast, unlike that observed in mammalian cells, would Pdi1 now become a “pro-secretory” factor if the M subunit was present? In mammalian cells, PDI is in excess of M. Perhaps in yeast a population of Pdi1 would complex with M and a separate population would still favor ERAD. Therefore,

the level of expression of the M subunit would have to be titrated to prevent depleting the yeast ER of all free Pdi1, which could potentially be lethal since *PDI1* is an essential gene.

4.2 YET FAMILY MEMBERS CONTRIBUTE TO APOLIPOPROTEIN B DEGRADATION IN YEAST

As ApoB is translated by the ribosome in the cytosol, a 27 amino acid signal sequence directs the ribosome-nascent chain complex to the Sec61 translocon, which is embedded in the ER membrane. The ribosome forms a tight seal with Sec61, allowing ApoB to cotranslationally translocate into the ER and preventing the protein from cytosolic exposure (section 1.2.2). If ApoB cannot assemble into a pre-VLDL in the ER, it is selected for ERAD and is retrotranslocated out of the ER and into the cytosol where it is degraded by the 26S proteasome; interestingly, unlike the majority of ERAD substrates, which are fully translated and translocated before ERAD, ApoB is targeted for ERAD co-translationally and maintains an interaction with the Sec61 translocon during degradation (section 1.4.2.2). In order for ApoB to be retrotranslocated out of the ER through the Sec61 translocon, the seal between Sec61 and the ribosome, that normally prevents cytosolic exposure, must be broken to allow cytoplasmic loops of ApoB to accumulate and be polyubiquitinated. The mechanisms for the change in interaction between Sec61 and the ribosome to facilitate degradation are poorly understood, but it is hypothesized that there are proteins that regulate this interaction. I identified two members of the Yet family of yeast proteins, Yet2 and Yet3, as potential regulators of the Sec61 translocon that may facilitate the retrotranslocation of ApoB through Sec61 for degradation and I have

started to examine the mammalian homologs of Yet2 and Yet3, BAP29 and BAP31 respectively. Several questions regarding how the Yet proteins facilitate ApoB degradation still remain. To understand how the Yet proteins mechanistically promote ApoB degradation I would like to ask whether the interaction of ApoB with the degradation machinery is altered in the absence of the Yet proteins. By determining which steps in ApoB ERAD are affected by the loss of these proteins I can place the function of the Yet proteins within the ApoB degradation pathway. Specifically, I would like to address the ubiquitination status of ApoB and determine whether the protein interacts with Cdc48/p97 in the *yet2Δ* and *yet3Δ* strains. To do this, I could immunoprecipitate ApoB29 from yeast cells that either express or do not express the Yet proteins and determine if Cdc48 is associated with ApoB by Western blot. Because the interaction between ApoB and Cdc48 is transient in nature (and may be difficult to detect by coimmunoprecipitation), the chemical crosslinker DSP could be added to spheroplasted yeast cells before immunoprecipitation, using a protocol that has been successful to immunoprecipitate ubiquitin ligases and their substrates (Gardner *et al.*, 2000). Additionally, the proteasome inhibitor MG132 could also be added to prevent degradation of ApoB, so that the interaction with Cdc48 will be favored. To detect polyubiquitinated ApoB, an ApoB immunoprecipitation and the detection of ubiquitin will be performed similar to that used to detect other polyubiquitinated ERAD substrates, such as CFTR (Ahner *et al.*, 2007) and ENaC (Buck *et al.*, 2010). Decreased ubiquitination and Cdc48 association in the absence of Yet proteins would support the idea that the Yet proteins facilitate retrotranslocation.

My hypothesis is that the Yet proteins aid in the retrotranslocation of ApoB and that ApoB is stabilized in their absence because it does not efficiently retrotranslocate from of the ER; alternatively, if the Yet proteins regulate the translocon, degradation of ApoB may be

prevented because it is not properly translocated into the ER, causing it to aggregate within the cytosol and thus preventing degradation. Although the Barlowe lab reported that CPY* and pof did not display translocation defects in the *yet1,2,3Δ* yeast strain (Wilson and Barlowe, 2010), to determine whether ApoB is translocated into the ER lumen there are two experiments that I could perform. First, I have shown using a cysteine modification assay (see section 2.2.4) that in wild type yeast cysteine residues within ApoB29 are oxidized to form disulfide bonds, a modification that occurs within the ER lumen. I could perform this assay using *yet2Δ* or *yet3Δ* strains and determine if ApoB has been subject to disulfide bond formation in the ER. A second method to determine whether ApoB is translocated into the ER could be to use an ER localized myc-tagged SNAP that would react with a benzylguanine labeled ApoB to form a covalent link between the two proteins, a method used by the Helenius lab to determine where BAP31 acts during SV40 infection (Geiger *et al.*, 2011). Using immunoprecipitation and Western blotting I could then determine if ApoB had translocated into the ER. Preliminarily, I have performed sucrose gradient fractionation with *yet3Δ* cells to examine ApoB localization (Figure 33). Based on these results, ApoB does co-localize with ER fractions, indicating that at least a population of ApoB is properly translocated when Yet3 is absent. Additionally, to determine if the Yet proteins facilitate retrotranslocation through their interaction with Sec61, I could make mutations in the Yet proteins that disrupt their interaction with Sec61 and determine if ApoB degradation is prevented as well.

As described in section 3.4, I will be repeating the pulse chase analysis of ApoB100 in McArdle cells when BAP31 has been knocked down and may also use siRNAs to silence BAP29 as well. If ApoB is stabilized when BAP31 and BAP29 are knocked down I could validate these results by using siRNAs targeted to the UTR of BAP29 or BAP31, which will knockdown these

proteins, and then rescue degradation of ApoB by transfecting the cells with either BAP29 or BAP31 constructs that lack the UTR. I would also like to perform indirect immunofluorescence to examine the localization of ApoB in mammalian cells when the BAP proteins have been silenced and I would expect to see an accumulation of ApoB within the ER.

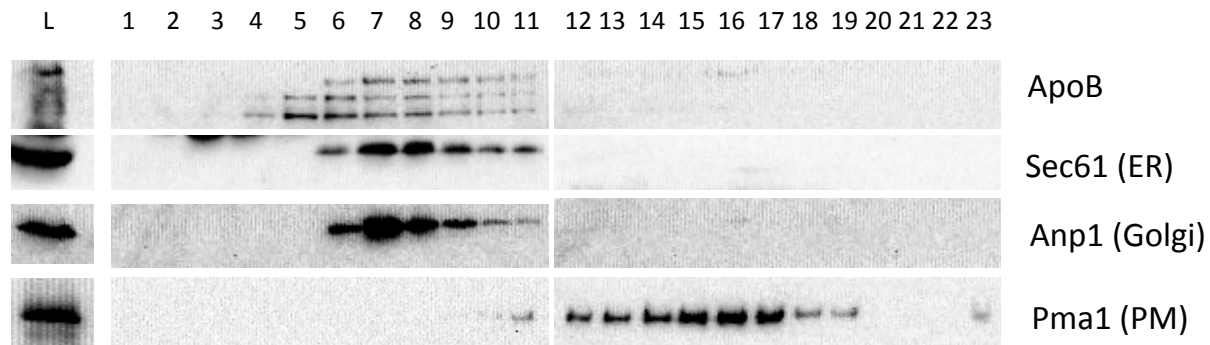


Figure 33. ApoB does not appear to be mislocalized in the absence of Yet3

Lysates from *yet3Δ* cells expressing ApoB29 were loaded onto a discontinuous sucrose gradient (20%-70%) in the presence of EDTA and centrifuged at 100,000xg for 19 hours. Fractions were taken from the top of the gradient and were subjected to SDS-PAGE and immunoblotted with anti-HA antibody to detect ApoB29. Anti-Sec61 antiserum was used as an ER marker. Anti-Anp1 antiserum served as a Golgi marker, and anti-Pma1 antiserum was used as a marker for plasma membrane proteins. A total of 1% of the lysate before gradient fractionation was also examined ("L").

If the Yet/BAP proteins regulate the translocon and loosen the change in the ribosome-translocon interaction, another question remaining is how these proteins “decide” that ApoB needs to be degraded and that the ribosome seal needs to be broken to facilitate exit from the ER. Do they associate with other proteins that pass along this information? Do they directly interact with exposed hydrophobic patches of ApoB when it is not assembled into a VLDL? Since these proteins have been shown to facilitate the retrotranslocation of other proteins, the mechanism promoting ApoB retrotranslocation may not be specific for ApoB. The proteins may act as general sensors for ER stress and facilitate the removal of proteins or viruses that could induce ER stress.

A final question that should be investigated with respect to ApoB ERAD is once ApoB is retrotranslocated into the cytosol, how is it kept soluble before degradation? Presumably ApoB is in an unfolded state with exposed hydrophobic patches in the cytosol. It is known that the heat shock proteins Hsp70 and Hsp90 bind to ApoB in the cytosol and target it for degradation. Are these proteins sufficient to prevent ApoB aggregation, or are there other proteins that associate with ApoB and perform this function? One family of proteins that interacts with unfolded substrates in the cytosol to prevent aggregation are the chaperonin proteins, CCT/TRiC (Spiess *et al.*, 2004). A graduate student in the lab is currently investigating whether these proteins are involved in ApoB degradation. A second group of proteins that may perform this function are the Get proteins. In yeast, the Get complex, composed of Get4, Get5, and Sgt2, shields transmembrane domains of tail-anchored proteins from the cytosol while they are shuttled to the ER membrane (Chartron *et al.*, 2012); thus, the Get complex could potentially interact with the hydrophobic domains of ApoB to keep it soluble. An analogous complex to the Get4-Get5-Sgt2

complex in vertebrates is TRC35/Ubl4a/SGTA, which also bind to another protein, Bag6, which has been shown to associate with ribosomes and interact with transmembrane domains of newly synthesized tail anchored proteins (Chartron *et al.*, 2012). This complex has been shown to not only bring tail-anchored proteins to the ER membrane but also to mediate the degradation of misfolded or mislocalized membrane proteins and retrotranslocated ER proteins (Minami *et al.*, 2010; Hessa *et al.*, 2011; Wang *et al.*, 2011). As such, this complex of proteins would make a good candidate for keeping ApoB soluble in the cytoplasm before degradation and would be quite interesting to investigate as an ApoB solubilization factor.

APPENDIX A

DOES THE STABLE POPULATION OF APOB LOCALIZE TO LIPID DROPLETS IN YEAST?

In yeast, approximately 50% of ApoB is stable (Figure 34), and is presumably protected from degradation and aggregation. I sought to determine whether the stable population of ApoB resided within the ER and could be kept soluble by chaperones, or if this population was differentially localized and protected from degradation by another mechanism.

When examining the localization of ApoB in yeast, I found that ApoB does not completely localize with ER markers. Sucrose gradient fractionation indicates that a population of ApoB resides in gradient fractions that are less dense than the ER and Golgi (Figure 35). When the gradient analysis are run in the presence of EDTA, the ribosomes are stripped from the ER membrane and the ER and Golgi both migrate to the same densities within the gradient (Figure 35A, lanes 5-12), while the plasma membrane migrates further into the gradient due to its higher density (Figure 35A, lanes 12-18). ApoB predominantly localizes to the same fractions as the ER and Golgi, although a small amount is consistently found in a fraction or two that is less dense than the ER and Golgi (Figure 35A, lane 4). To more definitively determine if ApoB co-migrates with the ER or with the Golgi I used a sucrose gradient poured in the presence

of magnesium. In the presence of magnesium ribosomes remain associated with the ER membrane and the ER density is much greater, causing it to co-migrate with the plasma membrane fractions (Figure 35B, lanes 13-19), while Golgi distribution is unaltered (Figure 35B, lanes 7-11). Interestingly, I found that when the ER and Golgi populations could be separated, a population of ApoB did co-migrate with the ER fractions, but a large population of ApoB did not shift with the ER and also peaked in less dense fractions than the Golgi (Figure 35B, lanes 4-6), suggesting that this population of ApoB is not localized to the ER or Golgi. Additionally, indirect immuno-fluorescence performed by a previous graduate student, Stacy Hrizo, suggested that while some ApoB co-localizes with the characteristic perinuclear staining of the ER marker BiP, ApoB also localizes to cytoplasmic punctae that resemble lipid droplets (Figure 36A). Lipid droplets are dynamic organelles that are ER-associated and provide a storage area for triacylglycerol and cholesteryl esters (Sandager *et al.*, 2002; Cermelli *et al.*, 2006). In mammalian cells a percentage of ApoB resides in lipid droplets and upon inhibition of the proteasome the concentration of ApoB residing in lipid droplets increases (Ohsaki *et al.*, 2006; Ohsaki *et al.*, 2008). I hypothesized that in yeast a population of ApoB may associate with lipid droplets since these are a rich source of lipids that could be loaded onto ApoB and the structure/organization of these organelles is very similar to that of lipoproteins into which ApoB assembles in mammalian cells.

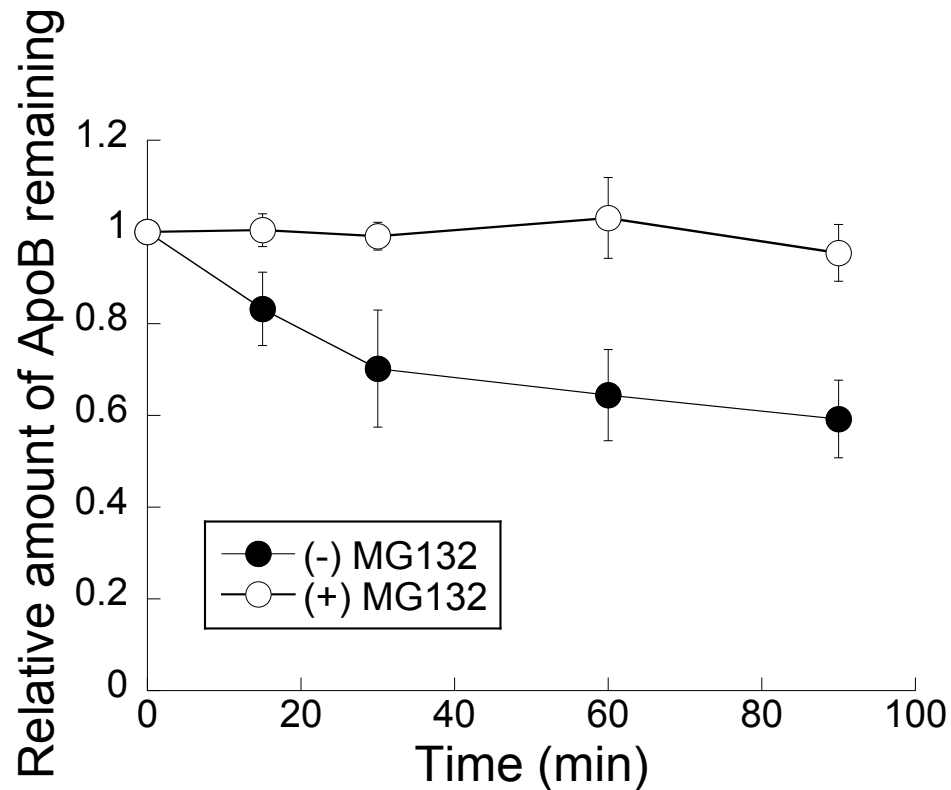


Figure 34. A population of ApoB remains stable during a cycloheximide chase

Cycloheximide chase reactions were performed in yeast strains expressing ApoB29 in either the absence (•), or presence (o) of the proteasome inhibitor MG132 (100μM) after a 20 minute pre-incubation with the drug. These strains were also deleted for *PDR5*, which facilitates the accumulation of small molecules. Chase reactions were performed at 30°C, and lysates were immunoblotted with anti-HA antibody to detect ApoB29. G6PD antiserum was used as a loading control (not shown). Data represent the means of 4 experiments, ± SEM.

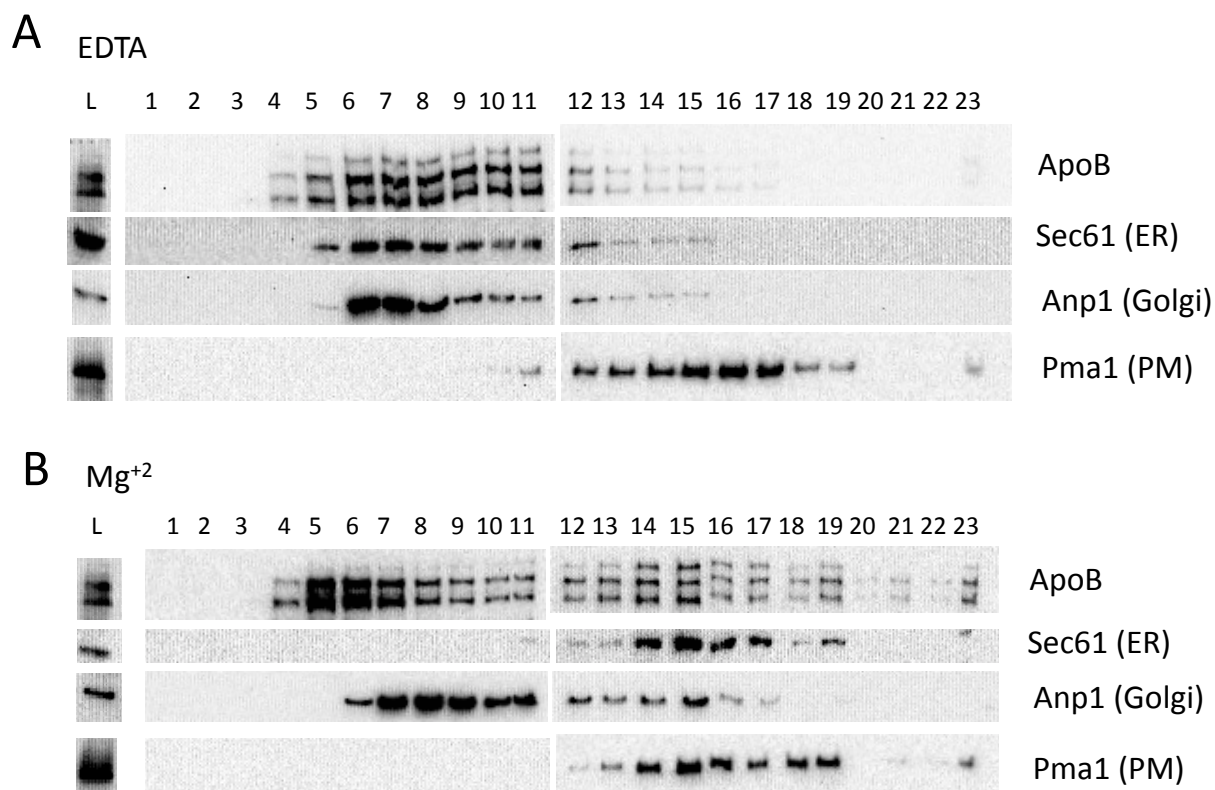


Figure 35. ApoB is present in sucrose gradient fractions that are less dense than the ER

Lysates from yeast expressing ApoB29 were loaded onto a discontinuous sucrose gradient (20%-70%) in the presence of (A) EDTA or (B) magnesium and centrifuged at 100,000xg for 19 hours. Fractions were taken from the top of the gradient and were subjected to SDS-PAGE and immunoblotted with anti-HA antibody. Anti-Sec61 antiserum was used as an ER marker. Anti-Anp1 antiserum served as a Golgi marker, and anti-Pma1 antiserum was used as a marker for the plasma membrane. A total of 1% of the lysate before gradient fractionation was also examined ("L").

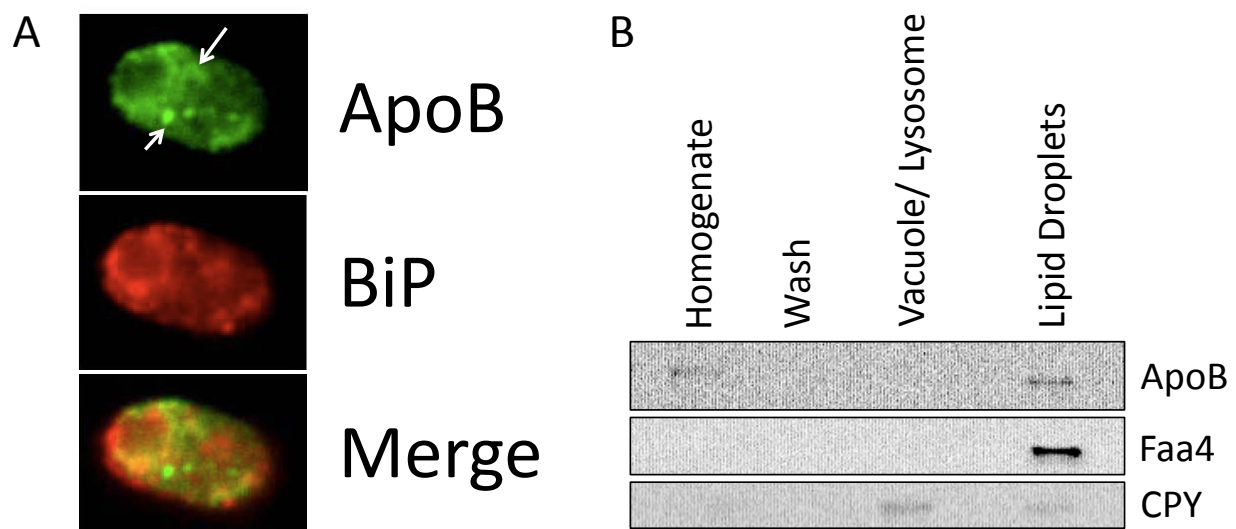


Figure 36. ApoB does not completely overlap with ER markers and can be biochemically isolated with Lipid Droplets

(A) BiP exhibits the typical perinuclear staining that is reported for ER luminal proteins. ApoB also exhibits the perinuclear staining, however intense staining of ApoB is observed in portions of the cytoplasm that do not overlap the ER staining (see white arrows). (B) Lipid droplets were isolated from spheroplasts expressing ApoB. Fractions were subjected to SDS-PAGE and immunoblotted with anti-HA antibody. Anti-GFP was used to detect Faa4, a marker for lipid droplets, and anti-CPY anti-serum was used as a vacuolar marker.

To determine if ApoB is found in lipid droplets in yeast, I isolated lipid droplets using a previously published protocol (Zinser and Daum, 1995). I found that ApoB could be isolated with lipid droplet fractions (Figure 36B). Since one hypothesis is that in mammalian cells lipid droplets are a staging area for degradation (Ohsaki *et al.*, 2006), I asked whether ApoB is still degraded when lipid droplets are absent. I examined ApoB stability using a strain where all four genes responsible for lipid droplet synthesis in yeast were deleted (*are1Δ*, *are2*, *iro1Δ*, *dga1Δ*) (a gift from the Stymne lab at the Swedish University of Agricultural Sciences) (Sandager *et al.*, 2002). Cycloheximide chase analysis suggests that lipid droplets are not required for ApoB degradation in yeast since ApoB is degraded at wild type levels in the absence of lipid droplets (Figure 37). Interestingly, even in the absence of lipid droplets a population of ApoB remained stable and was not degraded, suggesting that there may be other mechanisms to protect ApoB from degradation in the absence of lipid droplets. To determine whether the degradation of ApoB is still proteasome dependent when lipid droplets are absent, and does not occur through another degradation pathway (e.g. autophagy), I examined ApoB stability in the lipid droplet deficient yeast strain (*are1Δ*, *are2*, *iro1Δ*, *dga1Δ*), which was also deleted for the drug pump Pdr5, with or without the proteasome inhibitor MG132. I found that in the absence of lipid droplets ApoB degradation is still proteasome dependent (Figure 38). These data suggest that lipid droplets are not a staging area for ApoB ERAD in yeast.

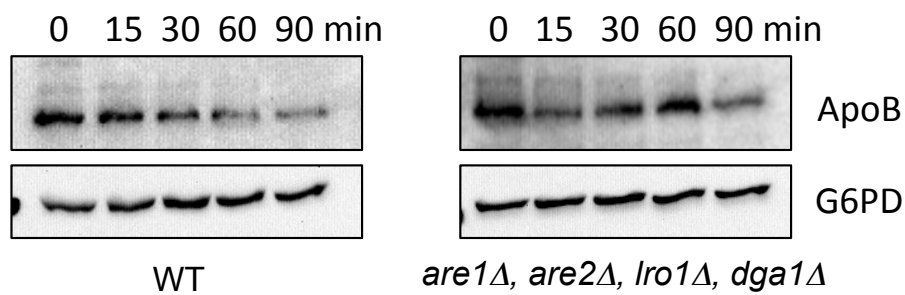
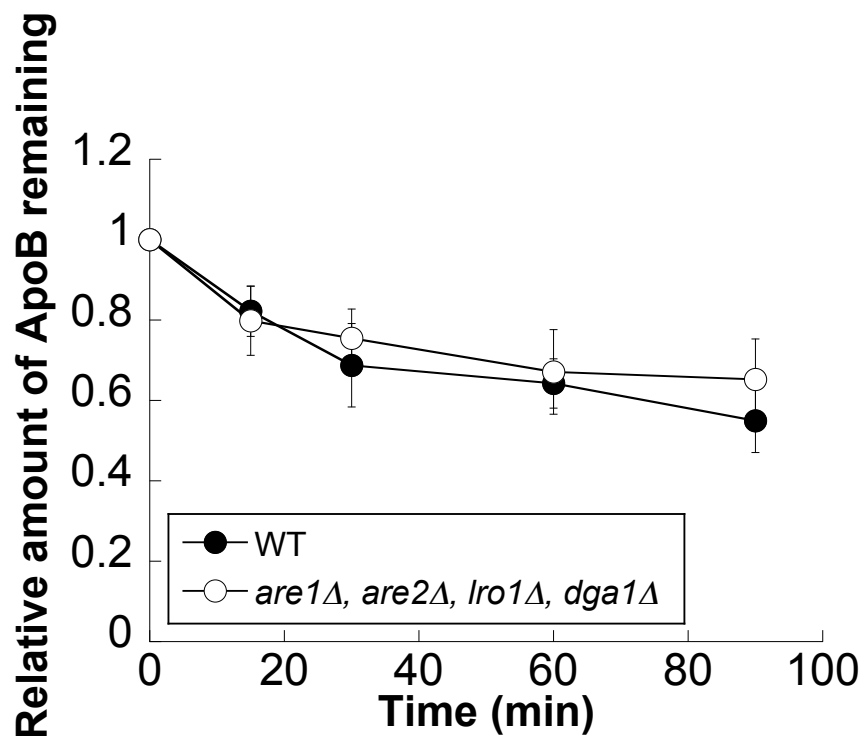


Figure 37. Lipid Droplets are not required for ApoB degradation

Cycloheximide chase reactions were performed in wild type (•), or *are1Δ are2Δ, lro1Δ, dga1Δ* (o) yeast strains expressing ApoB29. Chase reactions were performed at 30°C, and lysates were immunoblotted with anti-HA antibody and anti-G6PD antiserum was used as a loading control. Data represent the means of 6 experiments, \pm SEM.

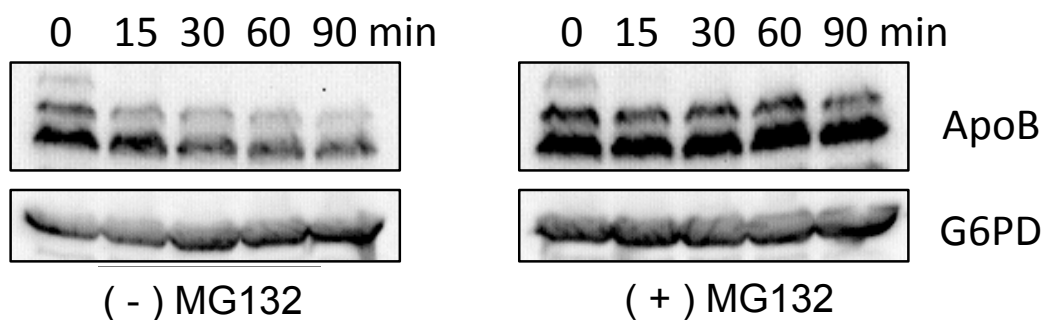
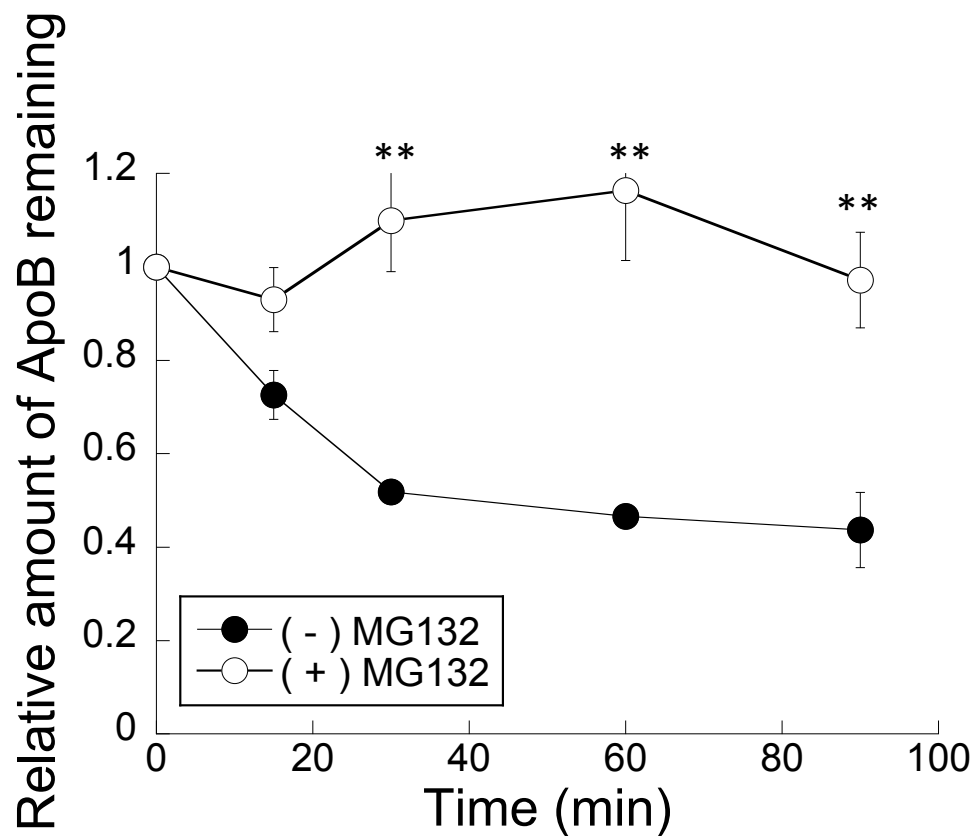


Figure 38. In the absence of Lipid Droplets ApoB degradation is still proteasome dependent

Cycloheximide chase reactions were performed at 30°C in *are1Δ are2Δ, lro1Δ, dga1Δ pdr5Δ* yeast strains expressing ApoB29. Cells were incubated with the proteasome inhibitor MG132 (100μM) (o) or a vehicle control (•) for 20 minutes prior to the addition of cycloheximide. Lysates were immunoblotted with anti-HA antibody and anti-G6PD antiserum was used as a loading control. Data represent the means of 4 experiments, ± SEM. Where indicated (**) $p < 0.01$

My data suggest that there are two populations of ApoB in yeast, a population residing in the ER that can be degraded and a stable population that resides in organelles that are less dense than the ER, presumably lipid droplets (Figure 39). To determine if the population of ApoB found in lipid droplets is protected from degradation Lynley Doonan, a graduate student in the lab, will continue this project and perform a number of experiments. To determine if the population of ApoB found in the less dense fractions of gradients is protected from proteasomal degradation, Lynley will perform cycloheximide chases of ApoB and load samples onto sucrose gradients to determine which population of ApoB disappears over time. Her preliminary results suggest that while the ER population of ApoB decreases after 60 minutes the less dense fraction remains. We would also like to know if the populations of ApoB change when proteasomal degradation is inhibited. If lipid droplets are protective environments for ApoB, I hypothesize that upon proteasomal inhibition the ER population of ApoB would shift to the lipid droplet fractions. To address this, Lynley will inhibit proteasomal degradation using MG132 and then use sucrose gradient fractionation to determine if more ApoB resides in less dense gradient fractions than ER fractions. Additionally, we would like to confirm that the cytoplasmic punctae observed by immunofluorescence and the less dense gradient fractions are lipid droplets. I have created a plasmid expressing Erg6, a protein known to localize to lipid droplets in yeast, fused to GFP that can be used for co-localization experiments and we will use this to determine whether ApoB resides in lipid droplets.

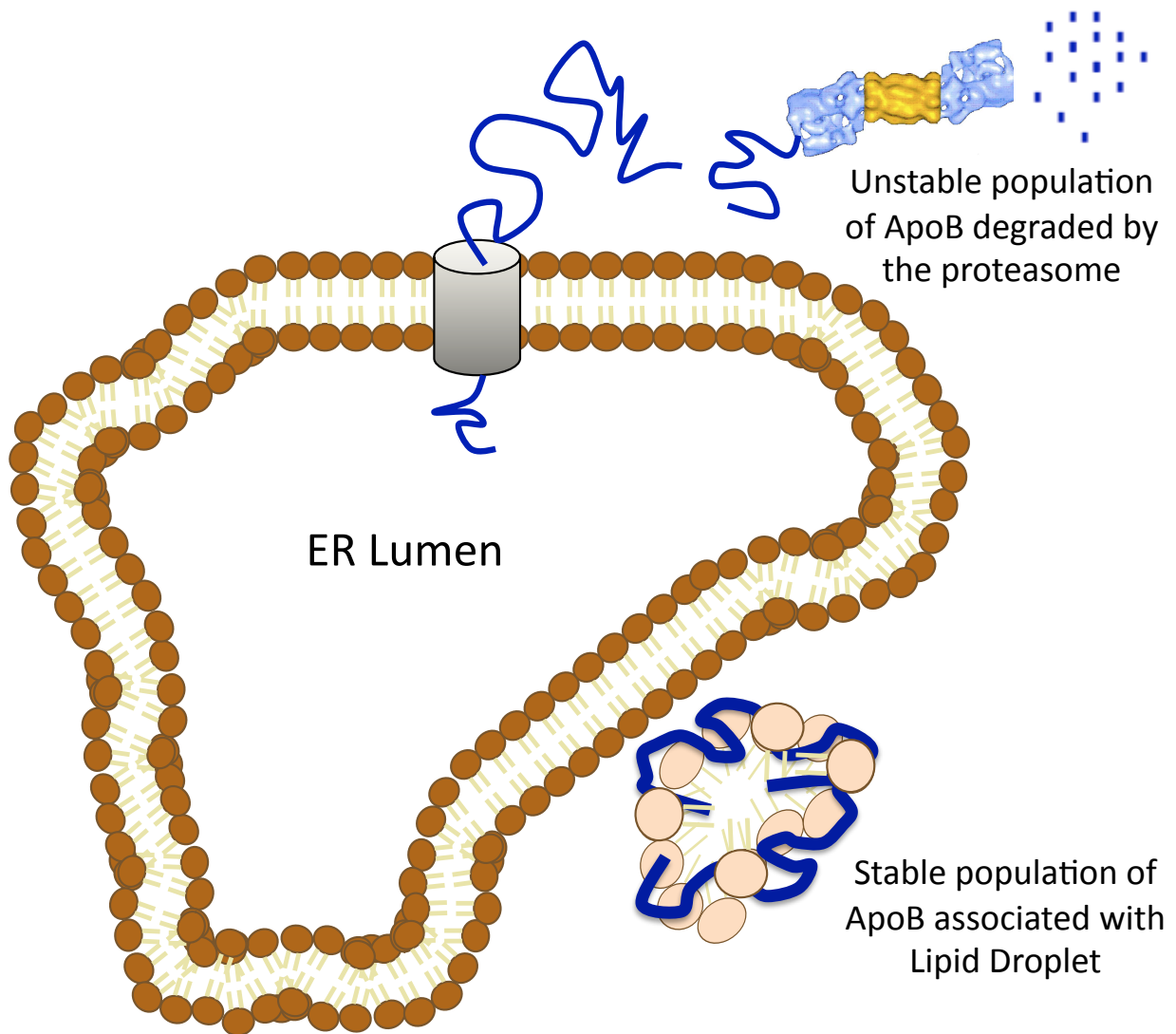


Figure 39. Model of the two populations of ApoB in yeast

A population of ApoB is found associated with the ER in yeast. This population is not actively or fully lipid loaded because the MTP complex is not present in yeast and is subject to degradation by the 26S proteasome. A second population of ApoB associates with lipids in the ER membrane due to its hydrophobic nature, and Lipid Droplets bud from this region of the ER forming separate organelles. ApoB assembled within Lipid Droplets resembles its lipoprotein-associated state and is protected from degradation and aggregation.

A.1 MATERIALS AND METHODS

A.1.1 Degradation Assay

Cycloheximide chases were performed as described in section 2.2.3.

A.1.2 Sucrose Gradient Fractionation

Forty ODs of yeast cells expressing ApoB29 were collected and resuspended in 10% STED Buffer (10mM Tris-HCl pH 7.6, 10mM EDTA, 10% sucrose) or 10% STMg Buffer (10mM Tris-HCl pH 7.6, 2mM MgCl₂, 10% sucrose) in the presence of protease inhibitors and 2mM DTT. Cells were lysed with glass beads (4 x 30 second pulses, with incubations on ice in between each lysis) and spun at 2000xg to remove cellular debris. The cleared lysates were loaded onto a discontinuous sucrose gradient (20%-70%) in the presence of STED or STMg Buffer and centrifuged at 100,000xg for 19 hours. Fractions were taken from the top of the gradient and were subjected to SDS-PAGE and immunoblotted with anti-HA antibody. Anti-Sec61 antiserum was used as an ER marker. Anti-Anp1 antiserum served as a Golgi marker, and anti-Pma1 antiserum was used as a marker for plasma membrane proteins. A total of 1% of the lysate before gradient fractionation was also examined.

A.1.3 Lipid Droplet Isolation

Lipid droplets were isolated from yeast expressing ApoB29 using a previously published protocol from (Zinser and Daum, 1995). Briefly, 2000 ODs of cells were spheroplasted and washed with potassium phosphate, pH 7.4, 1.2M sorbitol and then resuspended in breaking buffer (10mM MES-Tris pH 6.9, 12% Ficoll400, 0.2mM EDTA). The cells were homogenized for 45 strokes on ice with a loose Dounce homogenizer. A total of 9mL of breaking buffer was added to dilute the homogenate. The homogenate was spun at 4°C for 5min at 5000xg. The supernatant was moved into a thin walled ultracentrifuge tube and overlaid with 18mL of breaking buffer and centrifuged for 1hr at 4°C in a Beckman SW28 swinging bucket rotor at 28,000rpm. The thin floating layer at the top of the gradient was collected using a glass Pasteur pipette and the volume was brought up to 18mL in breaking buffer in a thick walled ultracentrifuge tube. This solution was overlaid with 18mL of Solution #2 (10mM MES-Tris, pH 6.9, 8% Ficoll400, 0.2mM EDTA) and centrifuged for 1hr at 4°C in a Beckman SW28 swinging bucket rotor at 28,000rpm. The top layer was collected and resuspended in Solution #3 (10mM MES-Tris pH6.9, 0.6M sorbitol, 8% Ficoll400, 0.2mM EDTA) and overlaid with Solution #4 (10mM MES-Tris pH6.9, 0.25M sorbitol, 0.2mM EDTA) and centrifuged for 30min at 4°C in a Beckman SW28 swinging bucket rotor at 28,000rpm. The top layer was collected and precipitated with a final volume of 10% TCA on ice for 15min and spun at 5000xg at 4°C for 5min. The pellet was resuspended in TCA sample buffer and subject to SDS-PAGE and Western blot analysis. CPY was used as a vacuolar marker, Sec61 was used as an ER marker, and FAA4 was used as a marker of lipid droplets.

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